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The Role of Erythropoietin in Ischaemia/Reperfusion Injury

Thesis submitted by

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Abstract

Background – Ischaemia/reperfusion accounts for a large proportion of fatalities in the developed world. Even if death is avoided, the patient suffers a deterioration in their quality of life. Erythropoietin (EPO) has been examined in clinical studies investigating its effect in anaemic chronic heart failure patients with any positive effect attributed to the correction of anaemia. Given the recent discovery of the EPO receptor on the myocyte surface, this thesis examined whether EPO could have a direct effect on the myocardium and limit ischaemia/reperfusion injury and the mechanism by which any protection occurs.

Methods and results – Using an isolated perfused rat model of ischaemia/reperfusion, we demonstrated that EPO could mimic preconditioning in a PI-3-Kinase (PI3K)-dependent manner. Administration of EPO at reperfusion limited infarct size by activation eNOS and could be abolished by inhibitors of NOS, PI3K and ERK 1/2. This thesis also showed that EPO could delay mitochondrial permeability transition pore opening (mPTP) in an oxidative stress myocyte model of mPTP opening, an effect that was suppressed by inhibitors of NOS and PI3K. A 3 week treatment of EPO reduced injury in a NOS-dependent manner that was independent of haematocrit. Finally this thesis demonstrated that administration of EPO as late as 30 minutes after commencement of reperfusion could still reduce infarct size.

Conclusion – This thesis demonstrated that EPO can be used in a variety of settings to elicit a protective effect against ischaemia/reperfusion injury. This variety of effective time points promises an important future role for EPO in the treatment of ischaemic heart disease.

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TABLE OF CONTENTS

ABSTRACT 2

ACKNOWLEDGEMENTS 3

TABLE OF FIGURES 8

LIST OF TABLES 12

LIST OF ABBREVIATIONS 13

CHAPTER 1 - INTRODUCTION 17

1.1 ISCHAEMIC HEART DISEASE 17
1.2 CELL DEATH 19
1.2.1 APOPTOSIS 21
1.3 PRECONDITIONING 24
1.3.1 PI3K 25
1.4 REPERFUSION 27
1.4.1 ERK 1/2 31
1.4.2 PROTECTION AGAINST REPERFUSION INJURY USING PHARMACOLOGICAL ACTIVATORS OF RISK 32
1.5 ERYTHROPOIETIN 34
1.5.1 EPO AND ERYTHROPOIESIS 34
1.5.2 STRUCTURE OF EPO 37
1.5.3 UPREGULATION OF EPO 39
1.5.4 THE EPO RECEPTOR 42
1.5.5 SIGNALLING PATHWAYS OF EPO 42
1.5.6 CLINICAL USE OF EPO 45

CHAPTER 2 - METHODS 46

2.1 GENERAL 46
2.2 CHEMICALS AND DRUGS USED 46
2.3 LANGENDORFF PERFUSION MODEL 46
2.3.1 EXCLUSION CRITERIA 48
2.3.2 DETERMINATION OF INFARCT SIZE 51
2.4 WESTERN BLOTTING 54

2.4.1 Tissue Collection 54
2.4.2 Protein Extraction 55
2.4.3 Protein Estimation 55
2.4.4 Polyacrylamide Gel Preparation 56
2.4.5 Protein Transfer 57
2.4.6 Immunoblotting 57
2.4.7 Quantification of Bands 58
2.5 H9c2 Culture **59**
2.5.1 Hypoxia/Re-oxygenation 59
2.5.2 Flow Cytometry 61
2.6 Preparation of Adult Rat Myocytes **64**
2.6.1 Confocal Microscopy 65
2.7 Statistics **66**

CHAPTER 3 - ERYTHROPOIETIN MIMICS PRECONDITIONING 67

3.1 Hypothesis, Aims and Protocol **67**
3.2 Experimental Protocols **68**
3.3 Results **70**
3.4 Discussion **73**
3.4.1 EPO Mimics Preconditioning 73
3.4.2 EPO Mimics Preconditioning through PI3K 75
3.4.3 EPO-induced Preconditioning is not ERK 1/2-dependent 77
3.4.4 Clinical Implications of EPO Preconditioning 78

CHAPTER 4 - EPO IN REPERFUSION INJURY 79

4.1 Introduction and Aims **79**
4.1.1 Mechanisms of Intervention 80
4.2 Experimental Protocol **81**
4.2.1 Western Blotting 83
4.3 Results **85**
4.3.1 Dose Response 85
4.3.2 Protein Kinase Inhibitors at Reperfusion 88
4.3.3 Western Blotting 92
4.4 Discussion **96**
4.4.1 Dose Response 96
4.4.2 Involvement of RISK Pathway 97
4.4.3 EPO at Reperfusion In Vivo 101
4.5 Conclusion **101**

CHAPTER 5 - EPO WITHIN A CELLULAR MODEL 103

5.1 Aims and Protocols **103**
5.1.1 Experimentation 104

5.2 RESULTS **107**
5.2.1 PROPIDIUM IODIDE 108
5.2.2 ANNEXIN V 109
5.2.3 VIABLE CELLS 110
5.2.4 THE ROLE OF MPTP OPENING IN EPO-MEDIATED PROTECTION 111
5.3 EXPERIMENTATION FOR MPTP OPENING **113**
5.4 RESULTS **114**
5.4.1 MPTP OPENING 114
5.4.2 EFFECT OF EPO AND L-NAME ON MPTP OPENING 116
5.4.3 THE INVOLVEMENT OF PI3K AND ERK 1/2 IN EPO-MEDIATED DELAY OF MPTP OPENING 117
5.5 DISCUSSION **118**
5.5.1 ATORVASTATIN INTERVENTION AT RE-OXYGENATION 118
5.5.2 EPO-MEDIATED DELAY IN MPTP OPENING 120
5.5.3 THE EFFECT OF U0126 121
5.5.4 INVOLVEMENT OF NOS IN EPO-MEDIATED DELAY IN MPTP OPENING 123
5.6 CONCLUSION **124**

CHAPTER 6 - THE EFFECT OF CHRONIC EPO TREATMENT ON THE MYOCARDIUM 125

6.1 INTRODUCTION AND AIMS **125**
6.2 EXPERIMENTAL PROTOCOL **126**
6.2.1 24 HOUR TREATMENT OF EPO 128
6.2.2 WESTERN BLOTTING 128
6.3 RESULTS **130**
6.3.1 HAEMATOCRIT 130
6.3.2 LANGENDORFF RESULTS 132
6.3.3 24 HOUR EPO GROUP 134
6.3.4 WESTERN BLOT RESULTS 135
6.4 DISCUSSION **141**
6.4.1 THE ROLE OF PI3K/AKT IN THE CHRONIC SETTING 142
6.4.2 THE ROLE OF NITRIC OXIDE IN CHRONIC EPO TREATMENT 144
6.4.3 EPO-MEDIATED CHRONIC PROTECTION IS NOT DUE TO RAISED HAEMATOCRIT 145

CHAPTER 7 - THE EFFECT OF EPO WHEN ADMINISTERED AFTER REPERFUSION 147

7.1 INTRODUCTION AND AIMS **147**
7.2 EXPERIMENTAL PROTOCOL **148**
7.3 RESULTS **150**
7.4 DISCUSSION **152**
7.4.1 A NEW PARADIGM FOR MYOCARDIAL SALVAGE? 154
7.4.2 CLINICAL IMPLICATIONS OF EPO-MEDIATED PROTECTION AFTER REPERFUSION 155

CHAPTER 8 - SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS 157

8.1 SUMMARY OF FINDINGS 157
8.2.1 EPO AS A PRECONDITIONING MIMETIC 157
8.2.2 EPO AT REPERFUSION 158
8.2.3 EPO WITHIN A CELLULAR MODEL 158
8.2.4 A MODEL OF CHRONIC EPO TREATMENT 159
8.2.5 EPO PROTECTS AFTER DELAYED ADMINISTRATION IN REPERFUSION 159
8.3 CONCLUSIONS 160
8.4 POTENTIAL CLINICAL IMPLICATIONS 160
8.4.1 ELECTIVE SURGERY 160
8.4.2 EMERGENCY INTERVENTION 161
8.4.3 GENERAL TREATMENT OF HEART DISEASE 161
8.5 LIMITATIONS AND FURTHER INVESTIGATIONS 161
8.5.1 PRECONDITIONING STUDIES 162
8.5.2 EPO AT REPERFUSION 162
8.5.3 EPO WITHIN A CELLULAR MODEL 162
8.5.4 CHRONIC EPO TREATMENT 163
8.5.5 EPO AT DELAYED REPERFUSION 163

PUBLICATIONS AND COMMUNICATIONS 165

Table of Figures

CHAPTER 1 - INTRODUCTION

Figure 1. 1, A simplified schematic of apoptosis 23

Figure 1. 2, The basic mechanisms involved in preconditioning 26

Figure 1. 3, Windows of therapy 29

Figure 1. 4, The Reperfusion Injury Salvage Kinase (RISK) pathway components. 31

Figure 1. 5, The regulatory effect of EPO on erythropoiesis 36

Figure 1. 6, Structure of EPO and its receptor 38

Figure 1. 7, The heme and HIF-1α oxygen-sensing mechanism by which EPO is upregulated. 41

Figure 1. 8, Ras/Raf signalling pathways of EPO 43

CHAPTER 2 - METHODS

Figure 2. 1, Schematic representation of the Langendorff perfusion apparatus used 49

Figure 2. 2, Langendorff perfusion apparatus used in experimentation 50

Figure 2. 3, Heart that has had the snare tied around the LAD 52

Figure 2. 4, Tetrazolium stained heart slices 53

Figure 2. 5, Planimetred heart slices of samples 54

Figure 2. 6, H9c2 cells in culture 59

Figure 2. 7, Hypoxic chamber 61

Figure 2. 8, Flow cytometer 62

Figure 2. 9, Representation of the mechanics of flow cytometry 63

Figure 2. 10, Confocal microscope 66

CHAPTER 3 - ERYTHROPOIETIN MIMICS PRECONDITIONING

Figure 3. 1, Representation of the change in Rate Pressure Product (RPP) throughout the experimental protocol 71

Figure 3. 2, The effect of ischaemic preconditioning (IPC), and of a 50ng/ml dose of EPO when administered as a preconditioning mimetic 72

CHAPTER 4 - EPO IN REPERFUSION INJURY

Figure 4. 1 Representation of the change in Rate Pressure Product (RPP) throughout the experimental protocol 86

Figure 4. 2, Dose-response curve measuring the infarct to risk ratio of 20-200 ng/ml doses of EPO given at reperfusion. 87

Figure 4. 3, Representation of the change in Rate Pressure Product (RPP) throughout the reperfusion experimental protocol 89

Figure 4. 4, The effect of a 50ng/ml dose of EPO when administered at reperfusion 90

Figure 4. 5, The effect of a 50ng/ml dose of EPO when administered at reperfusion solely, and with a 10μM dose of NOS inhibitor L-NAME 91

Figure 4. 6, The effect of a 50ng/ml dose of EPO on phospho-Akt levels in a model of regional ischaemia 92

Figure 4. 7, The effect of a 50ng/ml dose of EPO on phospho-eNOS levels in a model of regional ischaemia 93

Figure 4. 8, The effect of a 50ng/ml dose of EPO on phospho-Akt levels in a model of global ischaemia 94

Figure 4. 9, The effect of a 50ng/ml dose of EPO on phospho-ERK levels in a model of global ischaemia 96

CHAPTER 5 - EPO WITHIN A CELLULAR MODEL

Figure 5. 1, H9c2 cells in culture under 20x magnification light microscope 103

Figure 5. 2, The dot plot raw data produced by the flow cytometer 107

Figure 5. 3, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on propidium iodide 108

Figure 5. 4, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on annexin v 109

Figure 5. 5, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on cell viability 110

Figure 5. 6, Representation of the myocytes throughout confocal laser experimentation 115

Figure 5. 7, The effect of EPO (50ng/ml) and inhibition of NOS by L-NAME (10μM) on the time to depolarisation due to oxidant stress 116

Figure 5. 8, The effect of EPO (50ng/ml) and inhibitors of PI3K by wortmannin (Wort,100nM) and ERK 1/2 by U0126 (UO, 10 μM) on the time to depolarisation due to oxidant stress 117

Figure 5. 9, Confocal microscopy experiment using exactly the same protocol as mentioned in section 5.3, performed by an independent observer 122

CHAPTER 6 - THE EFFECT OF CHRONIC EPO TREATMENT ON THE MYOCARDIUM

Figure 6. 1, The effect of a chronic 3 week treatment of EPO on rat haematocrit 130

Figure 6. 2, The effect of the acute treatment with EPO on the rat haematocrit 24 hours after a single 5000U/Kg dose of EPO 131

Figure 6. 3, Representation of the change in Rate Pressure Product (RPP) throughout the experimental protocol 132

Figure 6. 4, The effect of a chronic treatment of EPO on infarct size 133

Figure 6. 5, The acute effect of EPO at a dose of 5000U/Kg given 24 hours prior to index ischaemia 135

Figure 6. 6 , The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total Akt and b) phospho-Akt levels within the myocardium 135

Figure 6. 7, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total PTEN levels and b) phospho-PTEN levels within the myocardium. 136

Figure 6. 8, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on total p42 levels and total p44 levels within the myocardium. **137**

Figure 6. 9, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on phospho-p42 levels and phospho-p44 levels within the myocardium. **138**

Figure 6. 10, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total eNOS levels and b) phospho-eNOS levels within the myocardium. **139**

Figure 6. 11, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on total iNOS levels within the myocardium. **140**

CHAPTER 7 - THE EFFECT OF EPO WHEN ADMINISTERED AFTER REPERFUSION

Figure 7. 1, Representation of the change in Rate Pressure Product (RPP) throughout the experimental protocol from stabilisation **150**

Figure 7. 2, The effect of EPO administration 15 minutes (EPO 15) and 30 minutes (EPO 30) after reperfusion **151**

List of Tables

CHAPTER 1 - INTRODUCTION

Table 1. 1, The cellular events associated with ischaemia in the myocardium **19**

Table 1. 2, Selection of studies that demonstrate EPO's protection in a variety of tissues **44**

CHAPTER 3 - ERYTHROPOIETIN MIMICS PRECONDITIONING

Table 3. 1, Characteristics of animals in the treatment groups **70**

CHAPTER 4 - EPO IN REPERFUSION INJURY

Table 4. 1, Characteristics of animals in the treatment groups **85**

Table 4. 2, Characteristics of animals in the treatment groups **88**

CHAPTER 6 - THE EFFECT OF CHRONIC EPO TREATMENT ON THE MYOCARDIUM

Table 6. 1, Characteristics of animals in the chronic treatment groups **132**

Table 6. 2, Characteristics of animals in the 24 hour treatment groups **134**

CHAPTER 7 - THE EFFECT OF EPO WHEN ADMINISTERED AFTER REPERFUSION

Table 7. 1, Characteristics of animals in the treatment groups **150**

LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis

%	percentage
ABC	ATP-binding cassette protein
ACE-I	angiotensin-converting enzyme inhibitor
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
Akt	cellular Akt/ protein kinase B
ANT	adenine nucleotide translocase
APAF-1	apoptosis protease-inducing factor-1
ATP	adenosine triphosphate
ATPase	ATP synthase
Bad	Bcl-X_L/Bcl-2-associated death promoter
Bax/BAX	Bcl-associated X protein
BCA	bicinchoninic acid
βcR	common beta receptor
Ca^{2+}	calcium ion
Caspase	cystein aspartate specific proteases
CCPA	2-chloro N^6 cyclopentyl adenosine
CEPO	carbamylated EPO
CFR	coronary flow rate
CK	creatine kinase
CsA	cyclosporin-A
Da	daltons
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetracetic acid

EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-tetra acetate
eNOS	endothelial nitric oxide synthase
EPO	erythropoietin
ERK	extracellular signal-regulated MAPK
FADH	flavin adenine dinucleotide
5-HD	5-hydroxydecanoic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK-3β	glycogen synthase kinase 3 beta
GST	glutathione-*S*-transferase
HK	hexokinase
HMG-CoA	hydroxyl-3-methylglutaryl-co-enzyme A
H_2O_2	hydrogen peroxide
HSP	heat shock protein
GPCR	G-protein coupled receptor
H^+	hydrogen ion/proton
IC_{50}	50% inhibitory concentration
IGF-1	insulin-like growth factor 1
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
JAK	janus kinase
JNK	c-Jun NHP_2 terminal kinase
K^+	potassium ion
K_{ATP}	ATP-sensitive potassium channel
kDa	kilodaltons
K_i	dissociation constant for inhibitor binding
LDH	lactate dehydrogenase
LAD	left anterior descending

L-NAME	N ω-nitro-L-arginine methyl ester
M-ABC	mitochondrial ATP binding cassette protein
Mg^{2+}	magnesium ion
MAPK	mitogen activated protein kinase
MEK	MAPK/Erk kinase
mK_{ATP}	mitochondrial K_{ATP}
mtNOS	mitochondrial nitric oxide synthase
mPTP	mitochondrial permeability transition pore
Na^+	sodium ion
NADH	nicotinamide adenine dinucleotide
nm	nanometres
NF-κB	nuclear factor kappa B
NHE	Na^+-H^+ exchanger
NO	nitric oxide
$\cdot O_{2^-}$	superoxide anion
·OH	hydroxyl radical
PARP	poly(ADP-ribose)polymerase
PDK	3-phosphoinositide-dependent protein kinase
P_i	inorganic phosphate
pH	pH
PI3K	phosphatidyl inositol 3-OH kinase
PKC	protein kinase C
PLSD	protected least significant difference
PTEN	phosphatase and tensin homolog
p70S6K	70-kDA ribosomal protein S6 kinase
p90RSK	p90 ribosomal S6 kinase
Raf	MAPK kinase
RISK	reperfusion injury salvage kinase

ROS	reactive oxygen species
RPP	rate-pressure product
RTK	receptor tyrosine kinase
SfA	sanglifehrin-A
SNAP	S-nitro N-acetyl penicillamine
STAT	signal transducer and activator of transcription
TK	tyrosine kinase
TMRM	tetramethyl-rhodamine methyl ester
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

Chapter 1 - Introduction

Despite the increasing prevalence of such diseases as AIDS and cancer, cardiovascular diseases remain by far the largest causes of mortality and morbidity. They cause one third of all deaths globally[1] with about half of these deaths attributed to coronary heart disease[2], making them one of the world's biggest killers. Within Europe, they are estimated to cost the European Union economy €169 billion per year, with coronary heart disease costing around €45 billion[2]. Clearly, coronary heart disease remains a huge problem in terms of deaths, debilitating conditions and economic burden on society. Therefore more research is required in order to try to limit death and improve the quality of life of those suffering from these diseases and, as such, thorough research resulting in effective treatment remains an imperative.

1.1 Ischaemic Heart Disease

Ischaemic heart disease is a major contributor of coronary heart disease and despite receiving much attention is still not understood completely. It is characterised as a disease that involves periods of no or low blood flow supply to the myocardium resulting in an inadequate oxygen delivery to meet demand. In individuals at rest, this lack of oxygen will usually be due to atherosclerotic lesions that obstruct or reduce blood flow in cardiac arteries preventing oxygenated blood reaching their intended tissue target. This limitation of oxygen supply has other implications, such as oedema, reduction in nutrient

availability and raised levels of potassium and sodium. Moreover an adenosine 5'-triphosphate (ATP) depletion and reduction in the pool of available adenylate[3] will not only affect the ATP dependent channels initially, but will also eventually lead to cell death in the case of continued paucity of ATP. An additional implication of reduced levels of oxygen is the occurrence of acidosis. Acidosis results on the one hand from lactate accumulation produced as a by-product of anaerobic glycolysis and and on the other from accumulation of CO_2 due to impaired blood flow. The level of lactate, initially increased in response to reduced levels of ATP[4], will eventually cause an inhibition of glycolysis. This metabolic wind-down is also manifest in the reduction in fatty acid oxidation[5] and oxidative phosphorylation, leading to diminished levels of NADH[6] as pyruvate is converted to lactate.

Irreversible cell death caused by ischaemia is thought to occur as a result of necrosis brought on by calcium overloading of the cell[7], which is discussed in greater detail in section 1.2.

All these elements may seem distinctly secondary in comparison with the problem of the oxygen debt caused by reduced blood flow. However, they all compound the difficulties faced when trying to prevent cell death.

Cellular Elements	Action as a result of Ischaemia
Oxygen Tension	↓
ATP	↓
Glucose	↓
Glycolysis	Initially rate increased, inhibited long-term
Fatty Acid Oxidation	Inhibited
pH	↓

Table 1. 1, The cellular events associated with ischaemia in the myocardium.

1.2 Cell Death

Although there have been several articles suggesting novel forms of cell death, there remain only 2 distinct recognised processes by which cells die, namely apoptosis and necrosis. Necrosis is an un-programmed method of cell death that has long been considered the method responsible for injury within the myocardium as a result of an ischaemic insult. During ischaemia it is thought that the reduction in oxygen brings about a switch to anaerobic respiration which has the effect of reducing stored levels of glycogen and the production of ATP. This lack of glycogen and ATP is thought to be the cause of ischaemia-induced necrosis within the cell [8]. The reduction in pH and accumulation of CO_2 associated with ischaemia tends to cause a sodium overload as the Na^+/H^+ pump attempts to discharge protons from the cell in exchange for sodium ions. This sodium overloading can itself lead to mitochondrial damage[9] but it can also lead to a catastrophic calcium overloading within the cell as a result of the exchange of this extra

sodium for calcium by the Na^+/Ca^{2+} transporter. This calcium has difficulty in being cleared from the cell as a consequence of dysfunction of the calcium ATPase transporter due to ATP depletion, thus exacerbating the problem of calcium accumulation.

Necrosis itself is characterised histologically by membrane disruption, such as to the cristae of the mitochondria and to the outer membrane, as well as by general mitochondrial swelling[10].

A study by Kajstura and colleagues examined the relative roles of apoptosis and necrosis within ischaemia/reperfusion-induced infarction in rats, and determined that apoptosis accounted for the greater part of the infarct size compared to necrosis[11]. This research was backed up in a study by Dumont and colleagues in which they examined the initiation of apoptosis subsequent to ischaemia/reperfusion by the use of the marker annexin v, suggesting that apoptosis was a major contributor to infarction[12]. However, a study by Ohno et al. found that the use of supposed end-stage markers of apoptosis (DNA laddering and TUNEL positivity), to measure apoptosis in a model of ischaemia/reperfusion in rabbits, demonstrated that a characteristic of necrosis, i.e. plasma cell disintegration, is also present[13]. Thus many studies that have used TUNEL or DNA ladder positivity as conclusive proof that apoptosis was the cause of cell death may actually have witnessed a hybrid form of cell death with both apoptotic and necrotic markers present. However, Yellon's group showed that inhibition of the caspase family, central to the apoptotic process, in particular caspases 3, 8 and 9, given at the point of reperfusion, substantially reduced infarct size[14]. Therefore, while it is difficult to distinguish precisely the manner of cell death responsible for much of the infarction

caused by ischaemia/reperfusion, according to classical definitions, the beneficial effect of inhibiting the apoptotic machinery suggests that prevention of apoptosis is a valid area of research in trying to limit injury in this setting.

From these studies it can be hypothesised that necrosis alone does not account for the totality of injury suffered as a result of ischaemia/reperfusion, but that apoptosis has a significant impact on cell death within the myocardium.

1.2.1 Apoptosis

Apoptosis is an energy-dependent process and therefore it is thought to be predominant once reperfusion has commenced[15, 16] due to the rapid restoration of ATP levels. Apoptosis can be initiated externally by cytokines, such as TNFα and Fas, or triggered internally by the release of cytochrome c from the mitochondria. Both of these pathways have the outcome of activating the caspase cascade which is central to the execution of apoptosis. These caspases are a series of enzymes that exist in an un-cleaved pro-form and are activated by cleavage at specific aspartate residues. As caspases are enzymes that can cleave at aspartate sites, once one caspase is activated it can initiate a proteolytic caspase cascade. Each caspase cleaves the pro-form of the next, subsequently activating it. The phenotypic endpoints of apoptosis reflect the cleavage targets of the caspases[17-20].:

1. Focal adhesion kinase – causing loss of adhesion,
2. Lamins – resulting in disassembly of nuclear lamina,
3. Structural proteins such as actin and gelsolin initiating blebbing

4. The activation of CAD endonuclease and inactivation of DNA repair enzymes, leading to DNA condensation and fragmentation.

The end result of apoptosis is the rounding of the cells into easily phagocytosed vesicles, which means that there is a reduced risk of inflammation.

It has been suggested that there are more ways for cells to die than merely apoptosis and necrosis; however none of the suggested additional methods have phenomena that are distinctively different from apoptosis and necrosis so it is difficult to class them as discrete processes. Instead, what seems likely is that a stimulus that triggers the initiation of the apoptotic pathway may become subverted into necrosis, possibly due to a depletion of ATP during ischaemia, for example[21]. A theory which tries to explain the switch between apoptosis and necrosis takes into account the relative rate of 'metabolic catastrophe' versus the speed of caspases and endonucleases activation. If the caspases and endonucleases are activated before metabolic dysfunction causes disruption of the cellular plasma membrane, apoptosis will occur; otherwise the phenotypic feature of cell death will be necrotic[22]. This apparent apoptotic initiation with seemingly necrotic end-points, as described earlier in the study by Ohno and colleagues[13], gives the appearance of an alternative process, but in actual fact cell death can be viewed as a spectrum with necrosis at one end of the scale and apoptosis on the other.

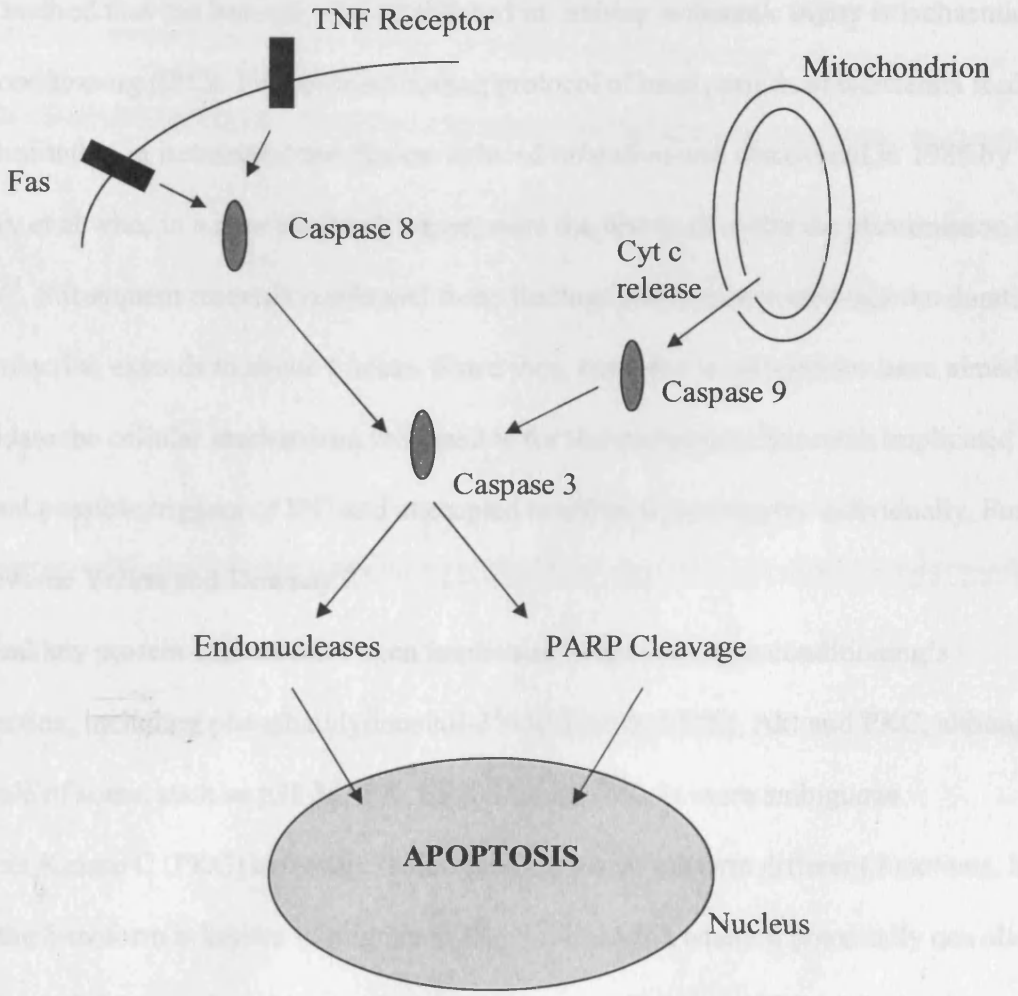


Figure 1. 1, A simplified schematic of apoptosis indicating initiation from external influences via TNF receptor and Fas, or internally by causing cytochrome c release from the mitochondria.

1.3 Preconditioning

One method that has become well established in limiting ischaemic injury is ischaemic preconditioning (IPC). The preconditioning protocol of brief periods of ischaemia leading to a limitation in ischaemia/reperfusion-induced infarction was discovered in 1986 by Murry et al who, in a now landmark paper, were the first to describe the phenomenon in dogs[23]. Subsequent research confirmed these findings and demonstrated that the duration of protection extends to about 4 hours. Since then, extended investigations have aimed to elucidate the cellular mechanisms responsible for this protection. Research implicated several possible triggers of IPC and attempted to utilise these triggers individually. For a review see Yellon and Downey[24].

Several key protein kinases have been implicated in mediating preconditioning's protection, including phosphatidylinositol-3'-OH kinase (PI3K), Akt and PKC, although the role of some, such as p38 MAPK, ERK 1/2 and JNK, is more ambiguous.

Protein Kinase C (PKC) has many isoforms that seem to perform different functions. In IPC the δ-isoform is known to migrate to the mitochondria where it potentially can alter the flow of electrons in oxidative phosphorylation or affect the mPTP inhibiting its opening[25], whereas the ε-isoform moves to the intercalated disk [26] and so possibly plays a role in intercellular communication [27]. A recent series of studies by Mochley-Rosen's group suggest that the δ-isoform is pro-apoptotic as selective inhibition of this isoform prior to ischaemia afforded protection in neonatal myocytes, adult primary myocytes and isolated organ perfusion models[28]. In contrast, the same group found that the ε-isoform was activated during IPC and that an ε-isoform knockout could not be preconditioned.

It has been suggested that PKC is downstream of PI3K in a signalling cascade which provides an additional method by which PI3K can implement its protective effect.

1.3.1 PI3K

PI3K was first implicated as a mediator of nerve growth factor's protection 10 years ago [29]. Since then much research has focused upon the mechanism by which PI3K imparts its protective effects. One crucial finding in this search was the discovery that PI3K causes the activation of Akt via the protein kinase PDK-1 which directly phosphorylates Akt[30-32]. Akt is a key component in imparting protection as it seems to have multiple targets that inhibit the apoptotic processes. For instance, it phosphorylates and, as a consequence, inhibits the pro-apoptotic factor Bad, preventing it from migrating to the mitochondria and causing cytochrome c release[33]. Akt also phosphorylates Caspase 9[34], forkhead transcription factors[35] and GSK 3β[36]. On the other hand Akt can phosphorylate, and thus activate, anti-apoptotic factors such as eNOS[37] and PKC[38]. All these actions individually and in conjunction have the overall result of preventing apoptosis and, importantly, Akt activation has been shown to be essential for the protection seen in preconditioning[39].

In implementing the protective outcome of preconditioning, finding the end effector has long been an ongoing target of research in this field. However, recently it has been demonstrated that the prevention of the opening of the mitochondrial permeability transition pore (mPTP) is a potentially important candidate as an effector of preconditioning[40].

This inhibition of pore opening has the effect of preventing cytochrome c release, calcium overload of the mitochondria and large-scale release of ROS, as well as the

collapse of the mitochondrial electrochemical gradient which results in ATP depletion. It is a matter of ongoing research, however, as to precisely how the 'survival' kinase pathway feeds into and/or influences the opening of the mPTP.

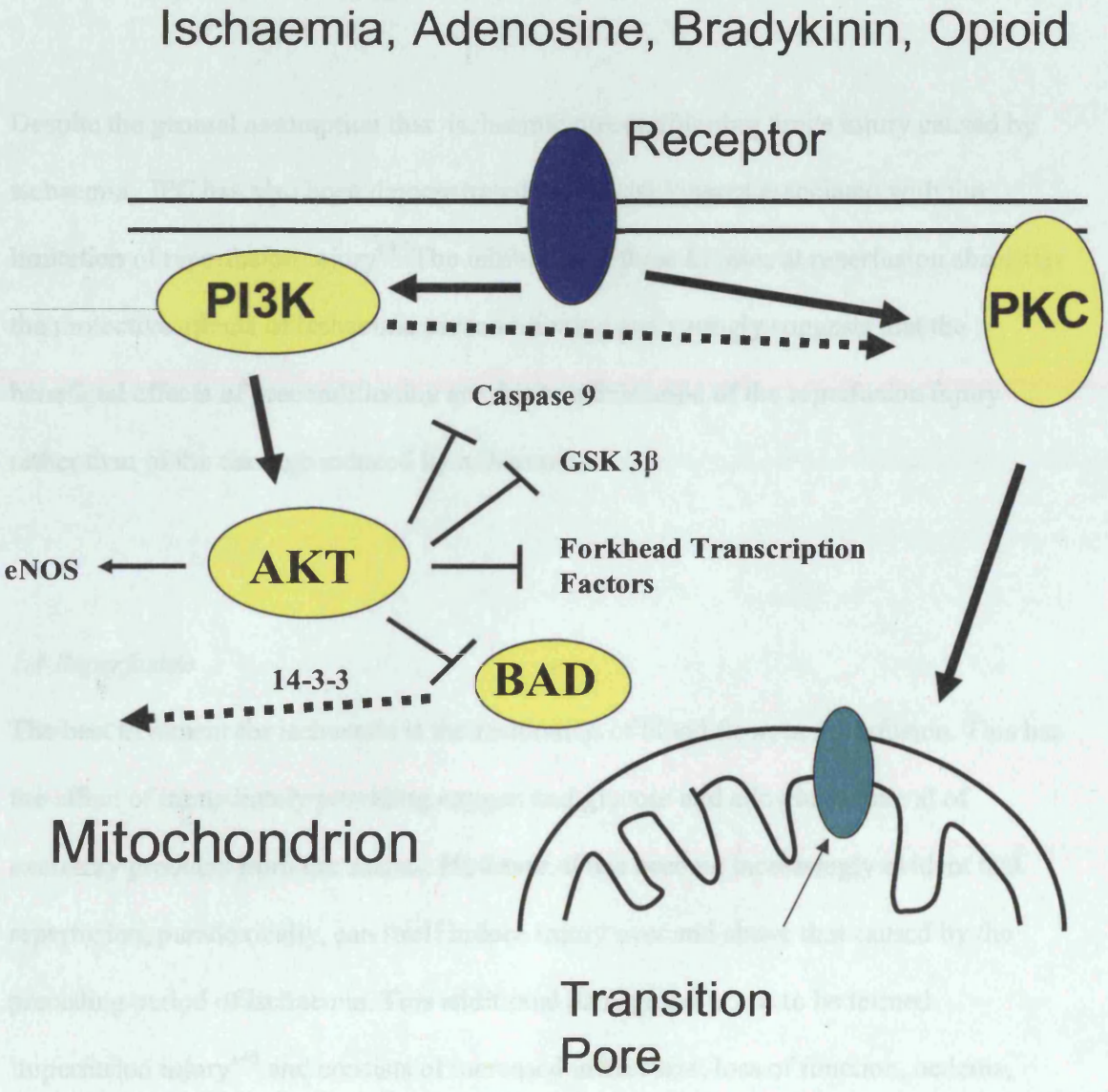


Figure 1. 2, The basic mechanisms involved in preconditioning

As a result, pharmacological interventions using adenosine receptor agonists[41], bradykinin[42], mito-K_{ATP} channel openers[43] and many others have shown that such interventions can mimic IPC's effects, i.e. that they appear to confer a "memory effect" even after they have been eliminated from the system.

Despite the general assumption that ischaemic preconditioning limits injury caused by ischaemia, IPC has also been demonstrated to activate kinases associated with the limitation of reperfusion injury[44]. The inhibition of these kinases at reperfusion abrogates the protective effects of ischaemic preconditioning and strongly suggests that the beneficial effects of preconditioning are due to a limitation of the reperfusion injury rather than of the damage induced by ischaemia.

1.4 Reperfusion

The best treatment for ischaemia is the restoration of blood flow, or reperfusion. This has the effect of immediately providing oxygen and glucose and allowing removal of excretory products from the tissues. However, it has become increasingly evident that reperfusion, paradoxically, can itself induce injury over and above that caused by the preceding period of ischaemia. This additional damage has come to be termed 'reperfusion injury'[45] and consists of increased infarct size, loss of function, oedema, damaged microvasculature and arrhythmias. The precise reason as to why this additional injury occurs is a matter of ongoing research with much attention being focused upon the role of reactive oxygen species (ROS) in causing, or triggering, this injury.

Experimentation suggested that free-radical scavengers could limit injury in a surgical model of ischaemia reperfusion[46]. Indeed, given that ROS release has been associated with the triggering of apoptosis[47], and that reperfusion injury has been associated with apoptosis as distinct from the necrosis attributed to cell death caused by ischaemia[48], it is entirely feasible that ROS is a major cause of apoptotic cell death resulting from reperfusion. It also seems that increased extra-mitochondrial calcium concentration enhances the cytochrome c-releasing potency of ROS[49]. This burst of ROS, in addition to an increase in mitochondrial calcium overload and correction of acidosis, are factors which favour the likelihood of mPTP opening with the consequence of mitochondrial membrane collapse, leading to ATP depletion, and necrosis. This is followed by cytochrome c release, resulting in initiation of mitochondria-dependent apoptosis[50]. Pharmacological inhibition of mPTP opening, by cyclosporine-A and by sanglifehrin-A administered at the point of reperfusion in an isolated rat model, confirms the role mPTP opening in initiation and mediation of cellular death[51-55]. Thus, as has occurred in the study of preconditioning, inhibition of mPTP opening has become a focus in limiting reperfusion-induced injury.

The difference in the mechanisms of cell death in ischaemia versus reperfusion seems potentially possible due to restored levels of ATP which are necessary for the apoptotic process, with an absence of ATP likely leading to a necrotic form of cell death. However, necrosis is not exclusive to ischaemia any more than apoptosis is to reperfusion.

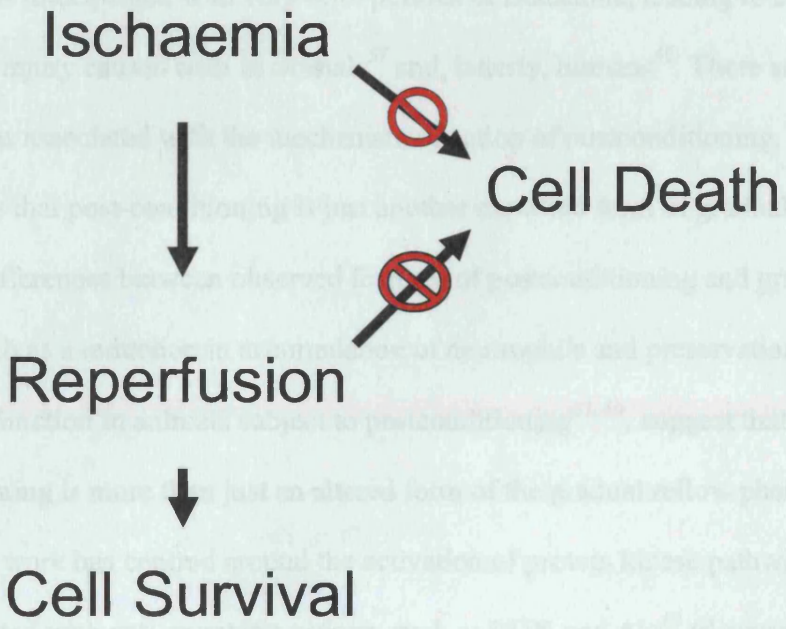


Figure 1. 3 Windows of therapy

Since injury can be caused by both ischaemia and reperfusion, it is possible that interventions can limit injury in both of these settings in a clinical scenario.

1.7 Kinase involvement in limitation of Reperfusion Injury

While it is important to re-introduce blood flow as rapidly as possible, too abrupt a reperfusion can cause an increase in severity of injury. It has been shown that a 'gradual reperfusion', i.e. where the flow initially was low but gradually increased, showed a

reduced infarct size compared to a group that had full flow restored from the commencement of reperfusion[56].

Recently, the concept of postconditioning has been introduced. This occurs when reperfusion is interspersed with very brief periods of ischaemia, leading to a significant reduction in injury caused both in animals[57] and, latterly, humans[58]. There are a number of hypotheses associated with the mechanism of action of postconditioning. One such hypothesis is that post-conditioning is just another modified form of gradual reflow. However, differences between observed features of postconditioning and gradual reflow[56], such as a reduction in accumulation of neutrophils and preservation of endothelial function in animals subject to postconditioning[57, 59], suggest that postconditioning is more than just an altered form of the gradual reflow phenomenon. More recent work has centred around the activation of protein kinase pathways that have been associated with anti-apoptotic actions, such as PI3K and Akt[60] (discussed in detail in sections 1.3.1 and 1.4.1). It is considered that both kinases act in a similar way to prevent cell death at reperfusion as they do as mediators of preconditioning. In addition to the PI3K-Akt pathway, certain other protein kinases have long been associated with infarct limitation, preservation of myocardial function and suppression of inflammation. These protein kinases form a mechanism recently termed the 'Reperfusion Injury Salvage Kinase' or RISK pathway[61]. This pathway can be utilised by pharmacological agents or other interventions, such as postconditioning, to successfully limit the extent of reperfusion-induced injury.

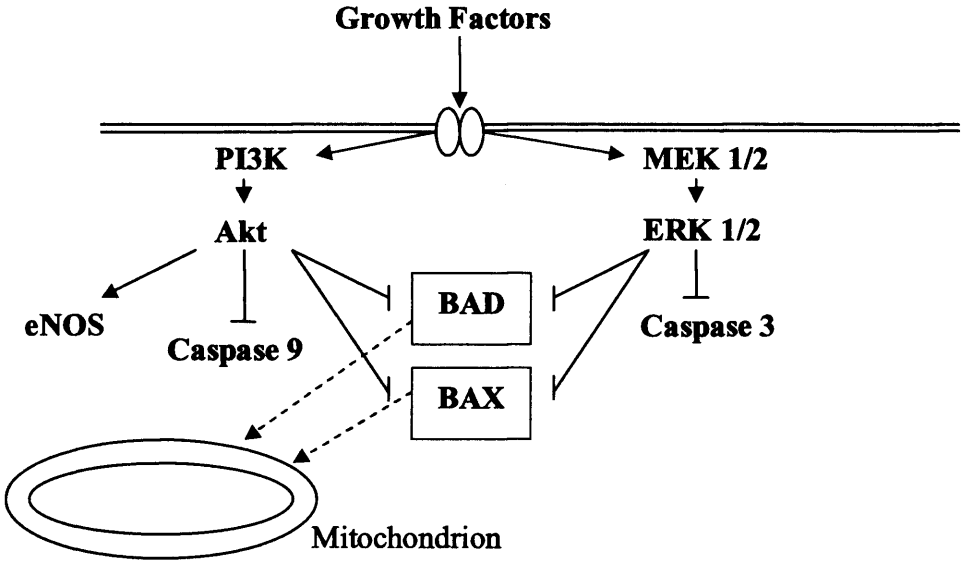


Figure 1. 4, The Reperfusion Injury Salvage Kinase (RISK) pathway components. Adapted from Hausenloy et al[61]

As can be seen from figure 1.4, the 2 major components of the RISK pathway are Akt and ERK 1/2.

1.4.1 ERK 1/2

ERK 1/2, also known as p42/44, belong to a family of Ser-/Thr- kinases also referred to as the Mitogen Activated Protein Kinases or MAPKs. Other members of this family include p38 and p46/54 or SAPK/JNK as they are also known. Whilst the other family members seem to be activated in response to some forms of stress, the activation of ERK

has been considered to be involved in growth and differentiation. Indeed, activation of ERK is usually necessary to bring about hypertrophy[62]. The MAPKs are activated by dual phosphorylation of a Thr-X-Tyr motif[63] in response to certain growth factors binding to their G-protein-linked receptors and activating the Ras-Raf pathway. ERK itself is activated by dual phosphorylation at Thr202/Tyr204 for ERK1 and Thr185/Tyr187 for ERK2, both of which can be phosphorylated individually. Although both sites must be phosphorylated to activate the kinase[64], there is not much information about whether there is any difference between p42 and p44 in the cell's response. Upon activation ERK translocates to the nucleus where it activates transcription factors, such as myc, elk and brf 1, known to be involved in differentiation and growth[65] by phosphorylating proline residues that reside next to serine or threonine residues[66].

Of more direct interest, with respect to the acute scenario of ischaemia/reperfusion, is the finding of Allen et al that ERK inhibits caspase 9 by phosphorylating Thr125[67]. Pharmacological inhibition of caspase 9 has been shown to limit reperfusion injury[14], thus providing a feasible mechanism of protection for ERK 1/2. In addition, ERK causes phosphorylation of the Ser 112 residue of BAD and thus inactivates it via p90rsk[68].

1.4.2 Protection against Reperfusion Injury using Pharmacological Activators of RISK

Agents that activate the RISK pathway have been used as potentially beneficial interventions in protecting against reperfusion injury. There have been many agents that have been studied and found to improve myocardial function and/or reduce the level of infarction. For example, Jonassen et al demonstrated that insulin could limit reperfusion-

induced injury in a p70s6 kinase- and PI3K-dependent manner in both a primary cell culture and an isolated heart perfused model[69]. Urocortin, a hormone related to the hypothalamic corticotrophin releasing hormone, was also shown to demonstrate protective properties when administered at the point of reperfusion. This protection was shown to be due to the activation of ERK 1/2. Furthermore, the ERK 1/2 inhibitor, PD98059, abrogated protection provided by urocortin thus indicating that ERK 1/2 was indeed a key component of protection[70]. In addition, a study by Brar et al, in neonatal cardiomyocytes, demonstrated that urocortin's reperfusion injury-limiting properties were PI3K dependent as the use of PI3K inhibitors abrogated the protection seen by urocortin administration at reoxygenation[71].

Despite being better known for its cholesterol-reducing properties, atorvastatin, an HMG-CoA reductase inhibitor, has also been shown to inhibit reperfusion injury in a PI3K-dependent manner[72]. Interestingly, a study by Bell & Yellon demonstrated that this protection was lost if an eNOS knockout strain of mouse was used, thus implying the significance of eNOS in atorvastatin-mediated injury[73]. The importance of these studies is that they indicate that atorvastatin exerts its action within the cell rather than acting as an agonist on external cell receptors as with the majority of compounds studied in the reperfusion setting.

Some adenosine receptor agonists have also been demonstrated to have a beneficial effect when administered at the point of reperfusion. The A1/A2a agonist AMP 579 is one such agonist that provided protection in an in vivo rabbit model of reperfusion-induced injury via an ERK 1/2-dependent mechanism[74]. Whilst the A1/A2 receptor agonist NECA has also been shown to reproduce the protection demonstrated by AMP 579, the failure of

adenosine itself and the A1 agonist, CGS21680, to similarly protect[75] exemplifies the complicated relationship adenosine receptor agonists have with protection in the reperfusion setting.

The aforementioned studies demonstrated how various agents, some already in clinical use, have an ability to limit reperfusion-induced injury and highlight the potential that other such agents may act in a similar way. One such agent is the glycoprotein erythropoietin (see below).

1.5 Erythropoietin

Erythropoietin (EPO) is a 30.4 kDa glycoprotein that has proven to be a vital hormone whose main function is considered to be the regulation of erythropoiesis, the production of red blood cells by bone marrow tissue.

1.5.1 EPO and Erythropoiesis

Erythropoiesis is the process by which pluripotent stem cells become, by various cellular intermediates, red blood cells (erythrocytes). It takes place in normal healthy humans within the bone marrow predominantly in the vertebrae, sternum and ribs, although the tibia and femur are also sites for erythropoiesis in persons under the age of about 25. The cells undergo a maturation process from pluripotential stem cells under the influence of different humoral factors, one of which is EPO.

There has been much research focusing on the characterisation of the mechanisms by which EPO promotes the erythropoiesis. Thus far two main processes have been described. Firstly, an increase in free EPO within the blood appears to cause an apparent

stimulation of haemoglobin synthesis in days 2 and 7 of the erythrocyte production process[76]. This increase in synthesis of a vital component of the mature erythrocyte allows the erythropoietic process to proceed. Secondly, EPO has been shown to prevent the programmed cell death of late erythroid precursor cells (Colony Forming Units-Erythroid, CFU-E) and erythroblasts in the bone marrow[77, 78]. These pre-cursors of erythrocytes are then allowed to proliferate and undergo the maturation process of erythropoiesis and form the mature red blood cell.

The EPO-derived inhibition of apoptosis in CFU-E is mediated through the up-regulation of Bcl-X_L via Stat 5[79], preventing cytochrome c release from the mitochondria. The activation of PI3-K by EPO[80] leads to a downstream activation of Akt, a known member of the 'survival kinase' pathways, providing an additional means by which EPO can prevent apoptosis, as discussed in section 1.2. An influx of Ca^{2+} into the cell via a voltage-independent ion channel, after EPO binds to its receptor, also seems to have a protective effect as it plays an important messenger role in the survival and proliferation of erythroid precursor cells[81].

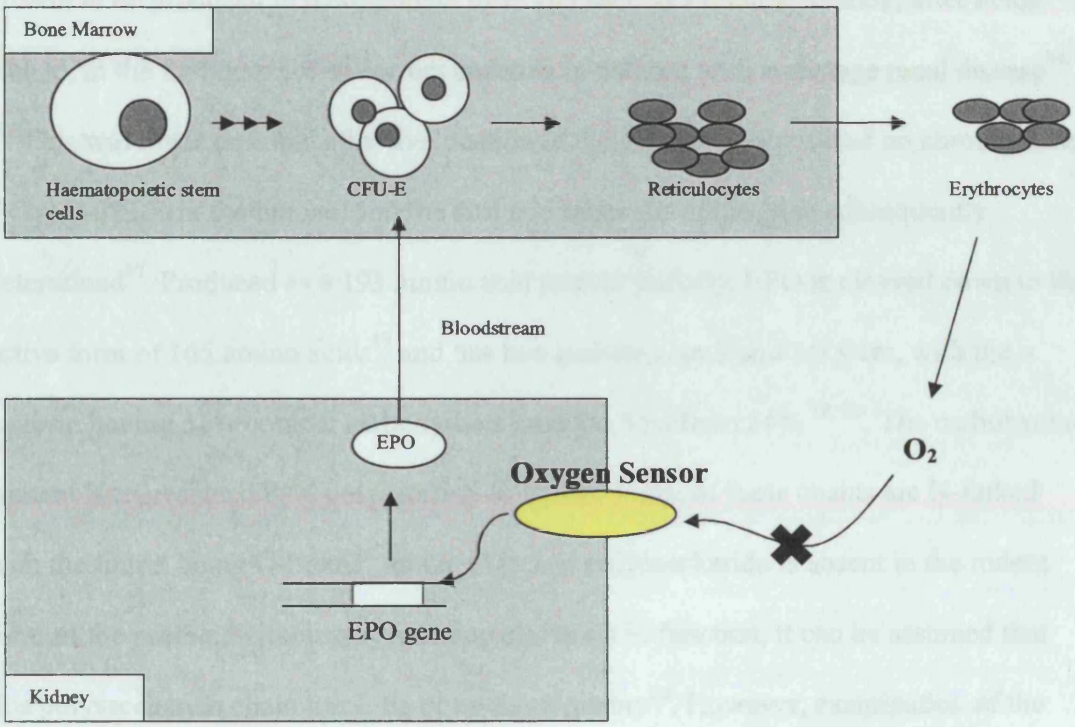


Figure 1. 5 The regulatory effect of EPO on erythropoiesis adapted from Sasaki et al[82]

In the erythropoietic process EPO seems to act in a self-regulating negative feedback loop by binding to red blood cells and thus no longer being 'free' and active to bind to its receptor. By this means, the more red blood cells there are in the bloodstream the more EPO is bound and active levels are diminished.

1.5.2 Structure of EPO

Purified for the first time by Miyake et al from the urine of patients[83], EPO was the first protein to be produced in recombinant form and used as a clinical therapy, after being cloned, in the first instance to correct anaemia in patients with end-stage renal disease[84, 85]. This was made possible after the location of the gene was discovered on chromosome 7 (7pter-q22)[86] in the human, and the size and sequence of the gene subsequently determined[87]. Produced as a 193 amino acid protein initially, EPO is cleaved down to its active form of 165 amino acids[88] and has two isoforms, an α and a β form, with the α isoform having 31% content carbohydrate, and the β isoform 24% [88, 89]. The carbohydrate content is represented by 4 polysaccharide chains. Three of these chains are N-linked with the fourth being O-linked. As the O-linked polysaccharide is absent in the rodent form of the protein, without any seeming alteration in function, it can be assumed that this polysaccharide chain has little or no functionality[90]. However, examination of the other 3 chains suggests that they are important for bioavailability and prevention of excretion[91]. One component of the polysaccharide chains is sialic acid that binds to galactose residues[92]. Interestingly, if the sialic acid is removed, EPO shows a greater affinity for its receptor in vitro increasing its activity by up to 5 fold[93]. However, its in vivo erythropoietic effect is lost as the protein is rapidly degraded by the liver[94], indicating that the chain and the sialic acid play a role in preventing degradation of EPO.

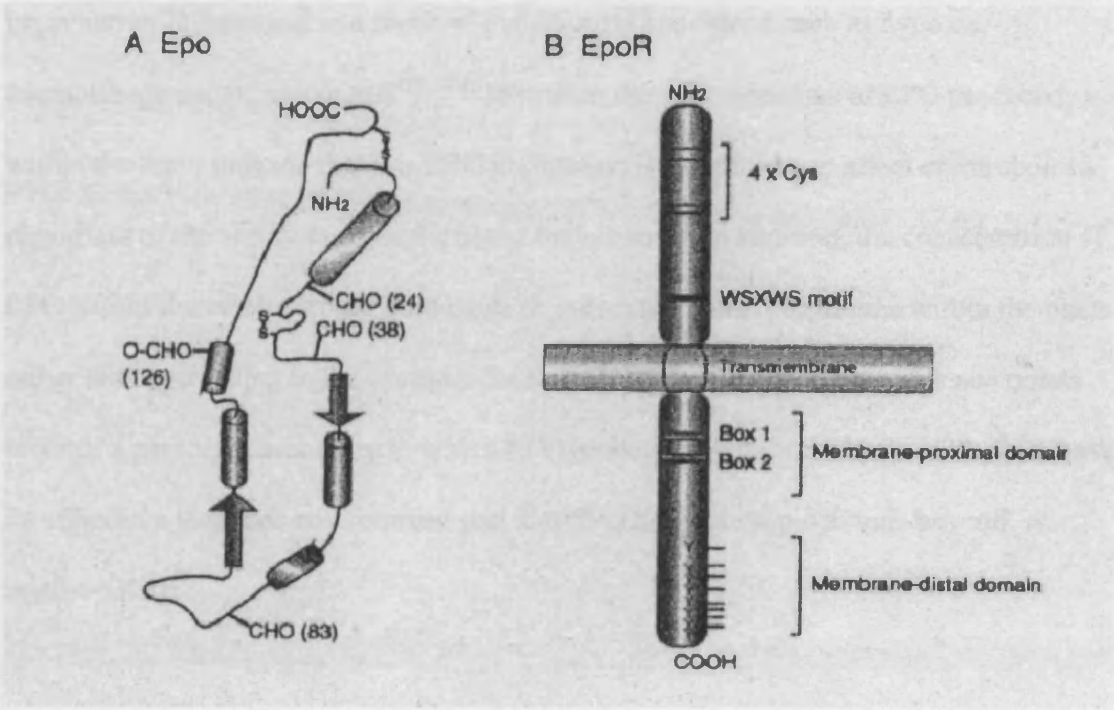


Figure 1. 6, Structure of EPO and its receptor from Sasaki et al [82]

Experimental nephrectomy in animals resulted in an absence of increase in serum levels of EPO in response to anaemia or administration of cobalt. This suggests that EPO is predominantly released from the kidney.[95, 96] This data was further supported by evidence that patients with renal failure have lower serum EPO levels. It is estimated that 70% of EPO is produced in the kidney from the juxtatubular cells with the other 30% being released from the liver. However, EPO mRNA has also been discovered in brain, spinal cord, retina, testes and spleen in significant quantities[97, 98]. Being 30.4 kDa in size, it is a matter of conjecture whether the molecule is able to cross the blood brain barrier and take its effect systemically rather than locally. Some studies do indeed suggest that it can cross the blood brain barrier[99-101], whereas others imply that EPO can only cross if the blood

brain barrier is damaged as a result of pathological conditions such as hypoxia, haemorrhage and/or meningitis[102, 103]. However, the concentrations of EPO produced within the brain indicate that this EPO production is insufficient to affect erythropoiesis regardless of the ability to cross the blood brain barrier. In addition, the concentration of EPO within the cerebrospinal fluid tends to reflect any effects of trauma within the brain rather than correlating to the systemic levels of EPO[104]. Therefore, the evidence points towards a paradigm according to which EPO production within the brain is likely to have its effect in a localized environment and that EPO has pleiotropic effects beyond erythropoiesis.

1.5.3 Upregulation of EPO

As EPO is a regulator of erythropoiesis it is natural that, when the system needs to increase the oxygen-carrying capacity of the blood in response to hypoxia, an increase of the EPO levels will occur. This phenomenon has been well known in examples such as high altitude, where the oxygen level is low [105], or dilated cardiomyopathy resulting from renal hypoxia[106]. In fact, EPO production has become so closely related to ischaemia that serum EPO levels have been considered as a potential prognostic marker in the case of clinically-controlled congestive chronic heart failure[107].

The increase in EPO levels can take place via a variety of oxygen-sensitive mechanisms. One such mechanism depends upon an oxygen-sensing system whereby low oxygen tensions cause the iron ion in haem to lose association by equilibrium with oxygen[108]. An artificial way to mimic this situation is to use cobalt, nickel or manganese to substitute for iron in the porphyrin ring of haem. The inability of these ions to bind oxygen ensures that

the haem group remains in a deoxy form[108, 109]. One suggested explanation by which the haem causes an increase in EPO production is that a deficiency in iron or a maintenance of haem in the deoxy form will cause a reduction in H_2O_2 within the cell. H_2O_2 in this scenario acts as a secondary messenger to inhibit Hyoxia Inducible Factor (HIF-1), a transcription factor associated with the upregulation of EPO[110]. Alternatively, it has been suggested that NAD(P)H Oxidase could act as an inverse oxygen sensor by producing ROS from oxygen that ultimately acts as a messenger to inhibit EPO gene transcription via H_2O_2, as described before[111]. Reduced oxygen tension, therefore, would reduce the level of free radicals and remove the inhibiting barrier to EPO gene transcription. Another proposed mechanism by which EPO is upregulated is by the stabilisation of Hypoxia Inducible Factor 1α (HIF-1α). This transcription factor is continuously manufactured but rapidly degraded by the proteosome to prevent its action. However, during periods of low oxygen tension the protein becomes stabilised by prolyl hydroxylases[112] and is able to migrate to the nucleus where it causes transcription of, amongst others, the EPO and VEGF genes by binding to the Hypoxia Inducible Enhancer (HIE)[113, 114].

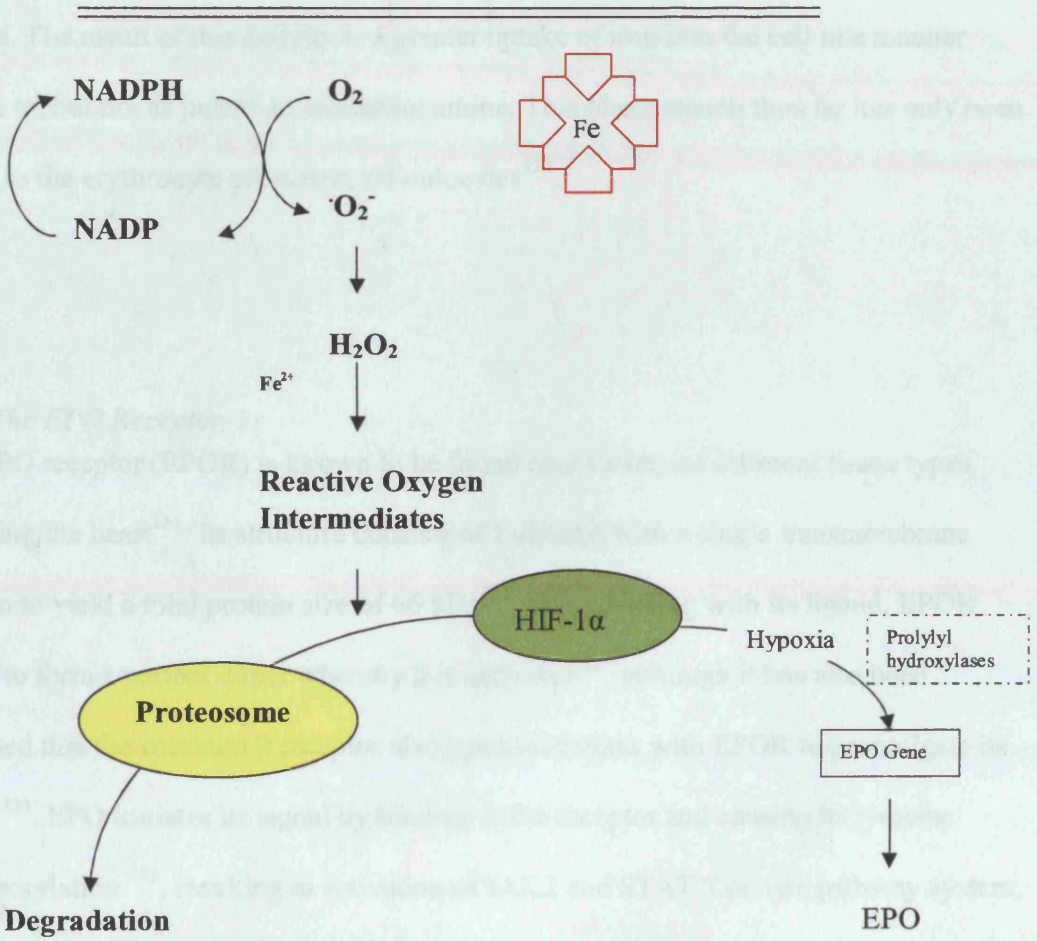


Figure 1. 7, The heme and HIF-1α oxygen-sensing mechanism by which EPO is upregulated. Adapted from Bunn et al.[115]

HIF-1α itself seems to act by binding to p300, which acts as a scaffolding protein enhancing transcription[116] and binds to the consensus sequence 5'- TACGTGCT -3' on the EPO gene[117]. Incorporated as part of this macromolecular assembly is the enhancer protein Hepatic Nuclear Factor 4 (HNF 4) binding to HIF-1β on the assembly and demonstrating enhancement of EPO gene transcription[118, 119]. EPO has also been demonstrated to modulate cellular iron metabolism by increasing the affinity of iron

receptors to their enhancer elements leading to an increase in iron receptors on the cell surface. The result of this activity is a greater uptake of iron into the cell in a manner similar to, but not as potent as, desferrioxamine. This phenomenon thus far has only been linked to the erythrocyte precursor, reticulocytes[120].

1.5.4 The EPO Receptor

The EPO receptor (EPOR) is known to be found on a variety of different tissue types including the heart[121]. Its structure consists of 1 subunit with a single transmembrane domain to yield a total protein size of 66 kDa[122]. Upon binding with its ligand, EPOR seems to form a perfect dimer whereby it is activated[123], although it has also been proposed that the common β receptor also heterodimerises with EPOR to promulgate its signal [124]. EPO initiates its signal by binding to the receptor and causing its tyrosine phosphorylation [122], resulting in activation of JAK2 and STAT 5 on one pathway system, and Ras/Raf as an alternative signalling pathway.

1.5.5 Signalling Pathways of EPO

Research has demonstrated that the binding of EPO to its receptor causes phosphorylation of tyrosine residues on the internal cellular domain of the EPOR [122]. Further activation of Gi protein [125] leads to downstream activation of the protein kinases PI3K and Akt, albeit in CHO Cells[126]. More recently EPO has been shown to cause activation of Jak 1/2, Stat 3, Stat 5A, PKC and p38 MAPK[127].

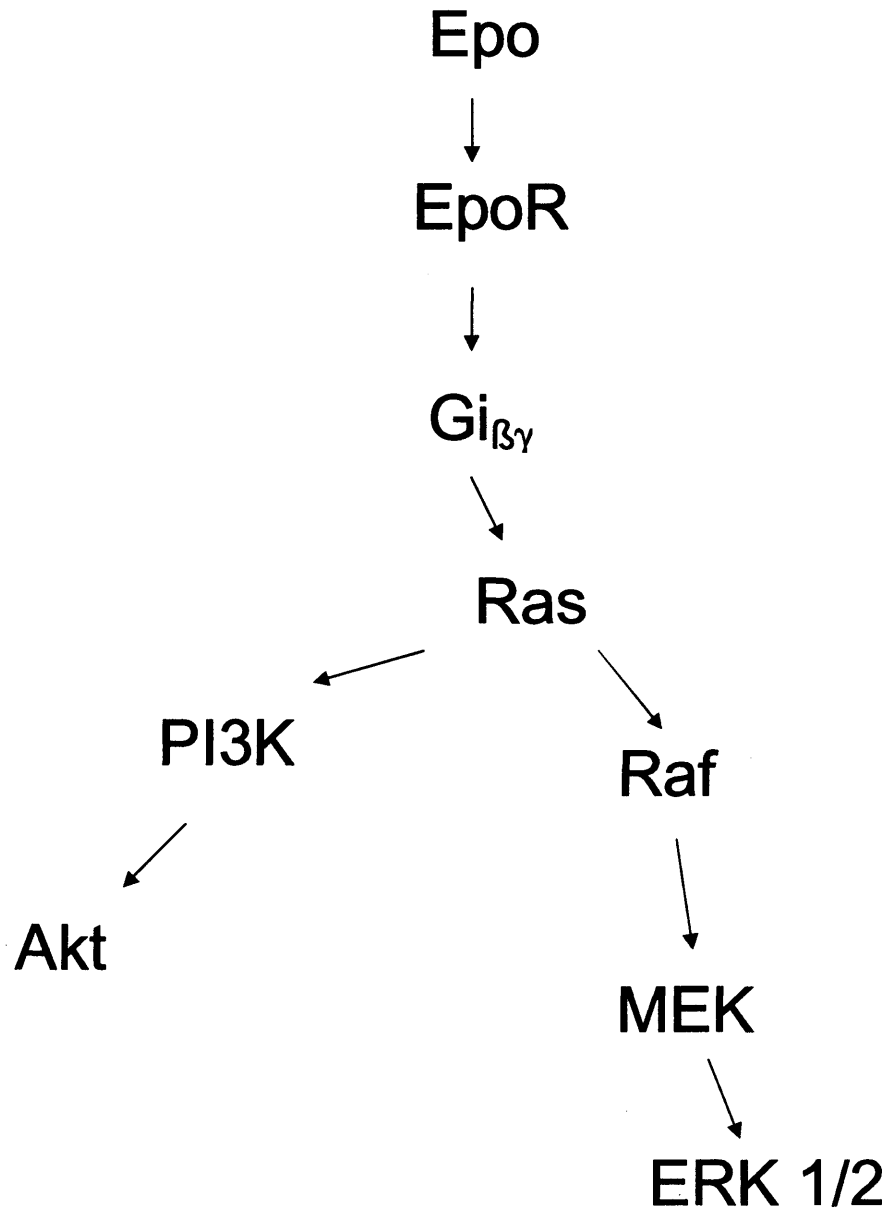


Figure 1. 8, Ras/Raf signalling pathways of EPO

As has been mentioned in section 1.3, activation of Akt and PKC prior to an ischaemic insult has been associated with limitation of ischaemia-induced injury. It was logical,

therefore, to hypothesise that EPO could limit injury when administered prior to or during ischaemia. As table 1.2 shows, EPO does indeed afford protection in a variety of tissues including neurons, brain and liver.

Tissue Type	Model	Signalling Pathways	Study
Neurons	Primary culture	Scavenging of free radicals	Sakanaka et al[128]
Brain	In vivo rat	N/A	Brines et al[99]
Brain/Neuron	In vivo rat Primary culture	ERK, Akt, Stat 5	Siren et al[129]
Brain	In vivo gerbils	Bcl x_L	Wen et al[130]
Fetal liver	In vitro rat	Lipid peroxidation	Solaroglu et al[131]
Cerebellar, hippocampal	Primary culture	Jak 2, Glutamate	Kawakami et al[132]

Table 1. 2, Selection of non-myocardial studies that demonstrate EPO's protection in a variety of tissues and mediated by various signalling pathways

With components including Akt, PKC, Jak 2 and Erk, it seems likely that EPO's protection is mediated through many of the same pathways associated with ischaemic preconditioning and successful pharmacological mimicry of preconditioning. During the

course of the research undertaken in this thesis, a number of studies investigating pre-ischaemic cardioprotection with EPO have been forthcoming. These studies will be discussed in the context of the work carried out in this thesis.

1.5.6 Clinical Use of EPO

EPO being a hormone that promotes erythropoiesis and the production of red blood cells, exogenous administration of recombinant EPO is, naturally, used as a clinical therapy predominantly in cases of chronic anaemia. Instances of anaemia tend to occur in cases of renal failure, as a result of renal carcinoma for example, and also in the setting of coronary heart failure that is associated with anaemia. As such, the use of EPO in chronic heart failure is not unknown and has been demonstrated to provide a beneficial effect by supposedly correcting the anaemia[133]. However, new research suggests that EPO activates certain protein kinases that have been associated with limitation of injury against ischaemia/reperfusion injury in many tissue types. This is the basis of the hypotheses that have led to an investigation of the ability of EPO to protect the myocardium from lethal ischaemia/reperfusion-induced injury and is the subject of this thesis.

Chapter 2 - Methods

2.1 General

Male Sprague-Dawley rats were obtained from Charles River (UK) and were treated in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986. All animals were acclimatised for a minimum of 5 days prior to use in a cage of no more than 5 animals containing access to water and standard pellet chow. Cages were maintained at 19-22°C with 55% ±10 humidity and subject to 12-hour light-dark cycle.

2.2 Chemicals and Drugs Used

EPO was supplied by Roche (Welwyn Garden City, Herts, UK). All constituents for the Krebs-Henseleit buffer were supplied by BDH Laboratory supplies (Poole, Dorset, UK). Triphenyltetrazolium chloride (TTC), all Western blot reagents and all tissue culture chemicals were provided by Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK). The BCA protein determination kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Tetramethylrhodamine methyl ester (TMRM) was supplied by Molecular Probes (Eugene, OR, USA). PBS was provided by Gibco Ltd (Paisley, Scotland, UK).

2.3 Langendorff Perfusion Model

The Langendorff model for perfusion of isolated hearts has been in use for over 100 years. Devised by Oscar Langendorff in 1895[134], this method of measuring cardiac function and infarction in vitro has become recognised as a robust reproducible model. The technique involves retrograde perfusion of a crystalloid buffer into the aorta under constant pressure. Langendorff himself used a fixed height reservoir to maintain constant

pressure. However, we used equipment from ADI instruments (UK) that employs pressure transducers to measure the perfusion pressure of the buffer entering the heart, and peristaltic pumps that control the rate of perfusion in order to maintain a constant perfusion pressure.

Male Sprague-Dawley rats (300-400g) were anaesthetised with sodium pentobarbital (50 mg/Kg ip). Heparin (300 IU) was administered concomitantly. Hearts were rapidly excised and placed in ice-cold buffer and mounted on a constant pressure (80 mm Hg) Langendorff-perfusion apparatus. They were perfused retrogradely through the aorta with modified Krebs-Henseleit buffer containing (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7 and glucose 12 and aerated with carbogen pH 7.3-7.5 at 37°C. Temperature was continuously monitored using a thermo-probe inserted into the pulmonary artery and maintained between 36 and 38°C. A latex balloon was introduced into the left ventricle through the left atrial appendage and inflated to give an end-diastolic pressure of 5-10 mm Hg. Regional ischaemia was achieved by tightening a 3/0 Mersilk suture around the main branch of the left coronary artery, using a round bodied curved surgical needle, and confirmed by a substantial drop in both left ventricular developed pressure and coronary flow rate. Hearts underwent 10 minutes of stabilisation, followed by 35 minutes of regional ischaemia. Loosening of the suture at the end of this period induced reperfusion as confirmed by a rise in the coronary flow, although coronary flow diminished throughout the 120 minute reperfusion period.

2.3.1 Exclusion Criteria

Hearts were excluded if a Rate-Pressure Product (RPP, heart rate multiplied by left ventricular developed pressure) did not achieve 17,000 beats/min.mmHg during stabilisation or the coronary flow fell below 10 ml/min. Hearts were also excluded if there was no drop in pressure and coronary flow on induction of ischaemia and a corresponding increase in coronary flow upon reperfusion. Finally, hearts were excluded if the risk volume was determined to be outside the range 0.3 cm^3 to 0.7 cm^3 or if the infarct to risk ratio fell beneath 5% as this level of injury was deemed to be due to mechanical means caused by the snare.

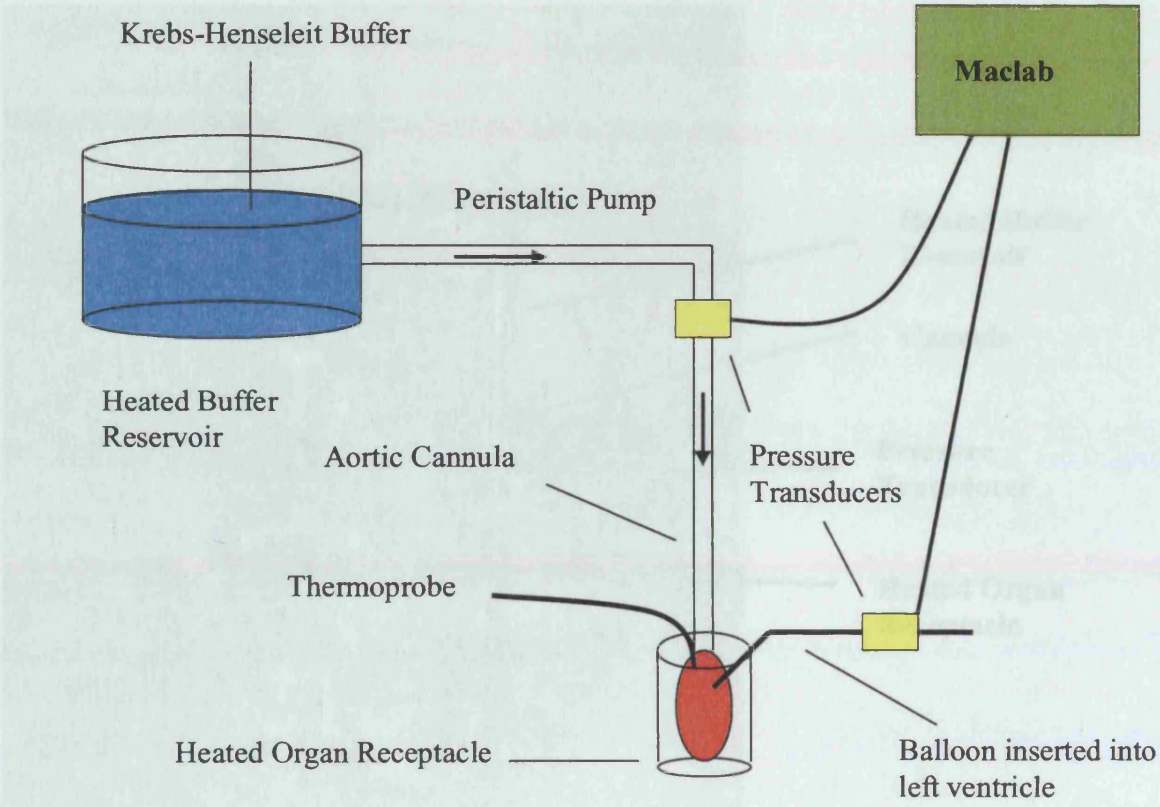


Figure 2. 1, Schematic representation of the Langendorff perfusion apparatus used

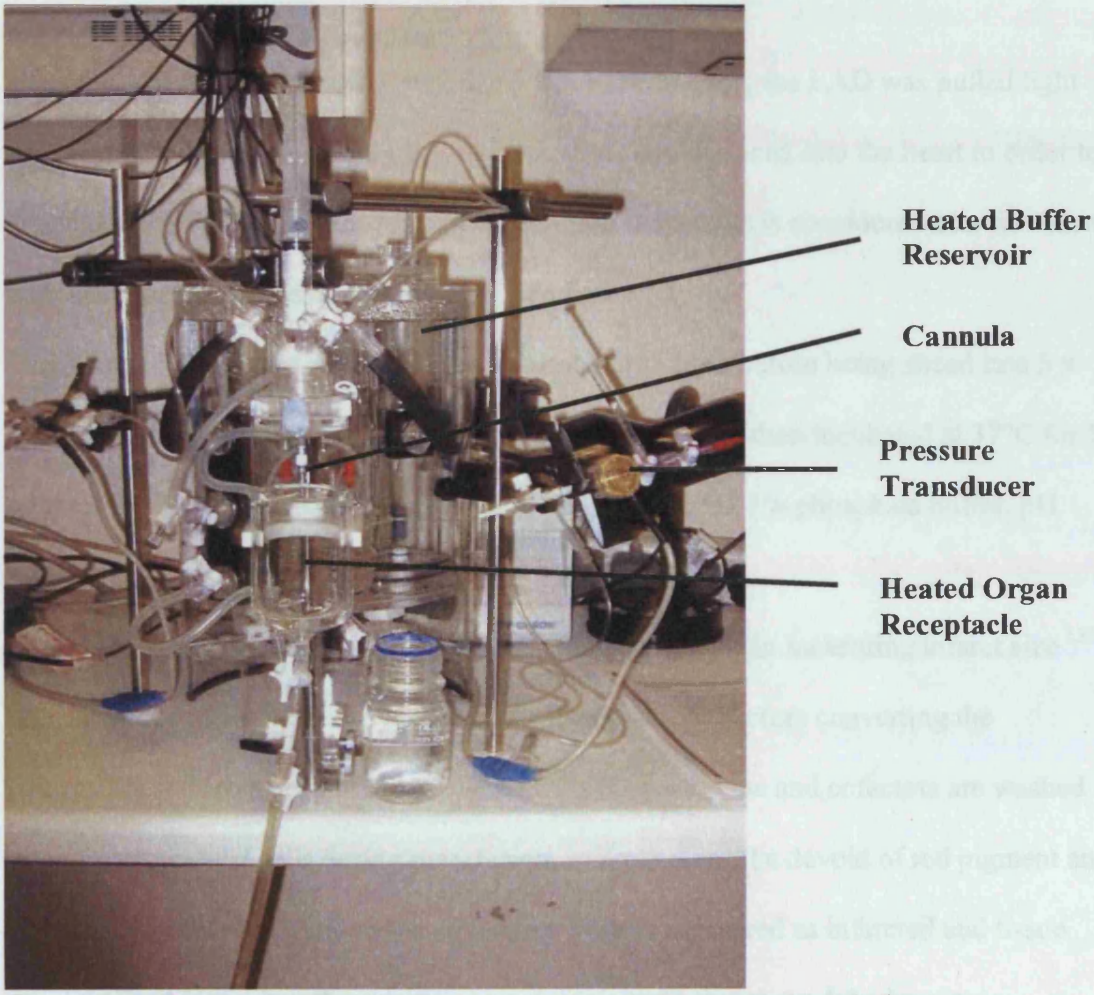


Figure 2. 2, Langendorff perfusion apparatus used in experimentation

2.3.2 Determination of Infarct Size

After the 120 minute perfusion period, the snare surrounding the LAD was pulled tight and a saline solution containing 0.25% Evans blue dye perfused into the heart in order to demarcate the risk area. The area of the heart that stains blue is considered non-risk tissue with unstained tissue being the risk area, see figure 2.4.

The heart is then frozen at -20° C for a minimum of 1 hour before being sliced into 5 x 2mm transverse sections from apex to base. The slices were then incubated at 37°C for 15 minutes in triphenyltetrazolium chloride (TTC) solution (in 1% phosphate buffer, pH 7.4).

TTC staining has become a standard and accurate technique in measuring infarct size [135]. The technique relies upon dehydrogenase enzymes and cofactors converting the tetrazolium salts to a red formazan pigment. As these enzyme and cofactors are washed away from the dead cells during reperfusion, infarction will be devoid of red pigment and will thus appear white. Any tissue appearing white is measured as infarcted and tissue appearing red is measured as viable. In order to ensure that these dehydrogenase enzymes and cofactors are washed away from the infarcted regions, there needs to be an adequate reperfusion period to avoid a false positive of infarcted tissue appearing to be viable [136]. For this reason a 2 hour period of reperfusion was used as part of a standard protocol.

Subsequent to TTC staining, heart slices were placed in 4% formalin (BDH, Poole UK) in saline for 24 hours. The formalin had the effect of bleaching the infarct area in order to demarcate it more clearly. The slices were then placed between 2 perspex sheets and

clamped by bulldog clips. An acetate sheet was placed over the top and the slices were traced blindly by an independent observer, the same person being used for all experiments to ensure consistency. A computerised planimetry package (Summa Sketch III, Summagraphics, Seymour, CT, USA) was used to determine the percentage of infarct size volume compared to risk zone and expressed as infarct-risk volume ration (I/R%), see fig 2.5.

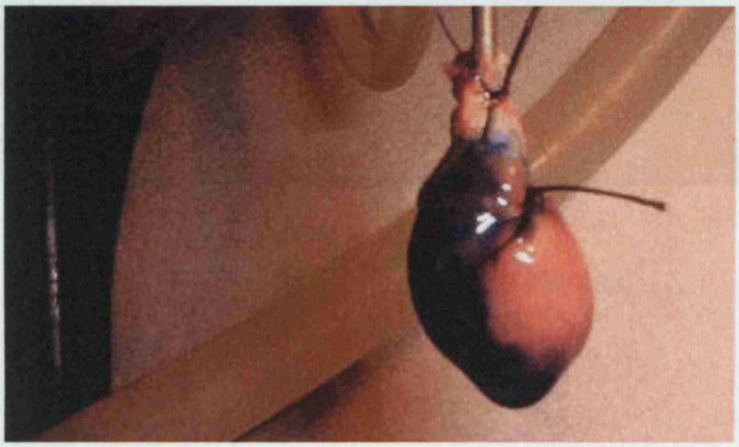


Figure 2. 3, Heart that has had the snare tied around the LAD and Evans blue dye injected down the aorta to demarcate the risk zone

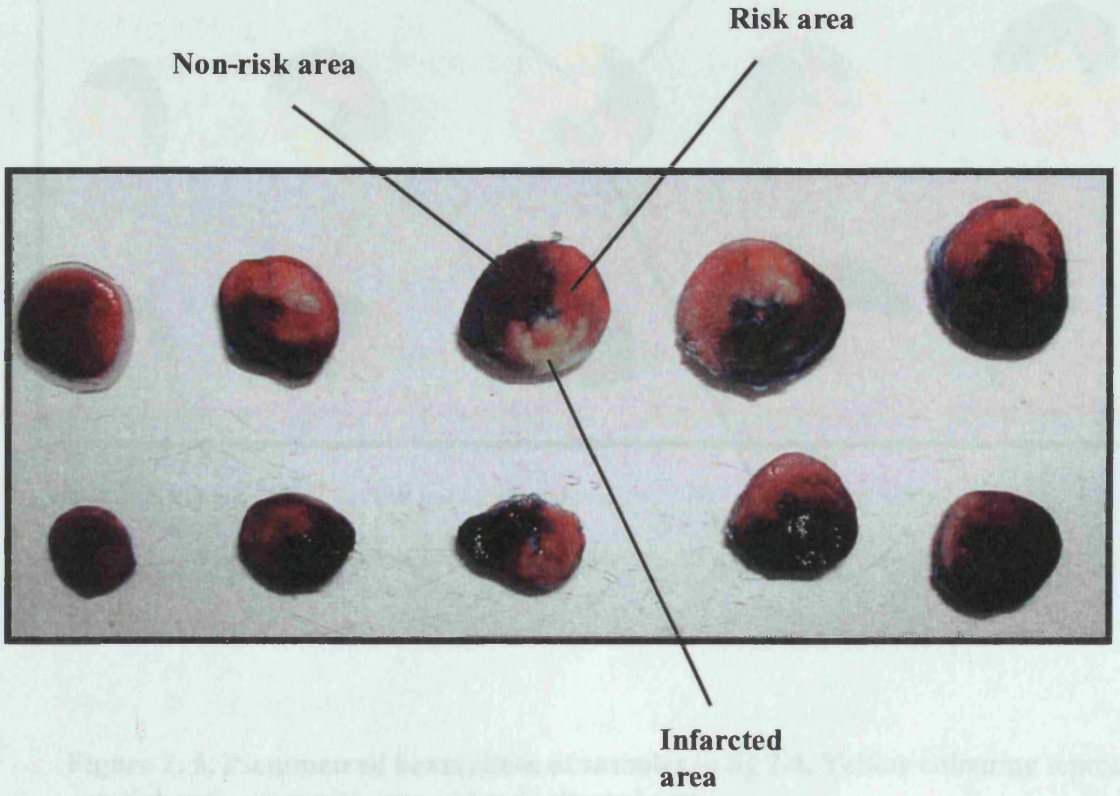


Figure 2. 4, Tetrazolium stained heart slices. Example of heart slices that have undergone TTC staining with the infarct area showing up white, viable risk tissue as red, and non-risk tissue staining blue.

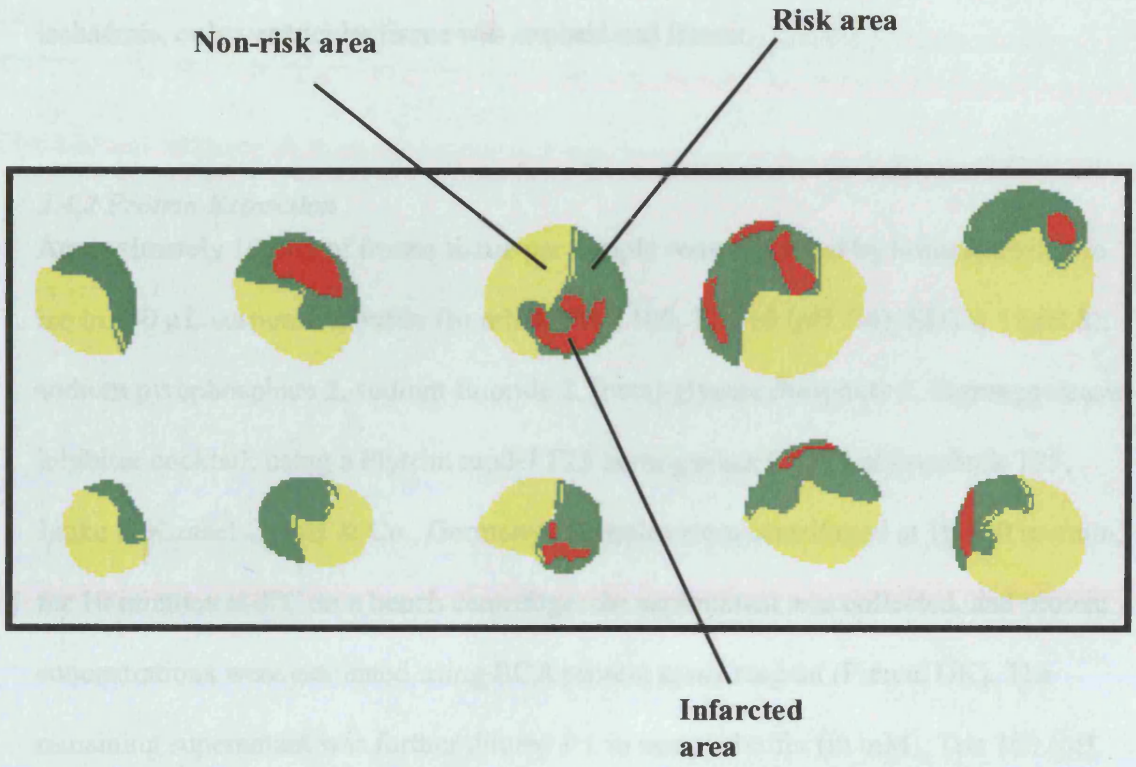


Figure 2. 5, Planimetred heart slices of samples in fig 2.4. Yellow colouring represents non-risk area, green risk area and red infarcted area.

2.4 Western Blotting

2.4.1 Tissue Collection

Rat hearts were prepared, mounted and underwent ischaemia/reperfusion as per the isolated heart preparation. Hearts were made ischaemic for 35 minutes followed by 15 minutes reperfusion, at which point the risk zone was identified using Evan's blue dye, excised, snap frozen in liquid nitrogen using pre-chilled tongs and stored at minus 80°C

for later use. When global ischaemia was used, i.e. the whole heart was subject to ischaemia, only ventricular tissue was excised and frozen.

2.4.2 Protein Extraction

Approximately 100mg of frozen tissue per sample were extracted by homogenising on ice in 250 µL suspension buffer (in mM): NaCl 100, Tris 10 (pH 7.6), EDTA 1 (pH 8), sodium pyrophosphate 2, sodium fluoride 2, [beta]-glycerophosphate 2, Sigma proteases inhibitor cocktail; using a Plytron model T25 homogeniser (IKA Labortechnik T25, Janke & Kunkel GmbH & Co., Germany). Samples were centrifuged at 10,000 rev/min, for 10 minutes at 4°C on a bench centrifuge, the supernatant was collected, and protein concentrations were estimated using BCA protein assay reagent (Pierce, UK). The remaining supernatant was further diluted 1:1 in sample buffer (in mM): Tris 100 (pH 6.8), DTT 200, SDS 2%, bromophenol blue 0.2%, and glycerol 20%, and subsequently boiled for 10 minutes at 100°C.

2.4.3 Protein Estimation

The protein concentrations for each sample were determined in order to be able to use the same amount of protein per sample on the Western blot.

The BCA protein assay works on the premise that cysteine, tryptophan, tyrosine and the peptide bond are capable of reducing Cu^{2+} in a copper sulphate reagent to Cu^{+}[137]. Bicinchoninic acid is then able to bind to Cu^{+} to give a purple colouration that is measurable in a linear manner at an absorbance of 562 nm. The protein content of the

samples can then be measured by comparison to a standard curve (20-2000 μg/ml) using bovine serum albumin (BSA) as the standard protein to give a concentration for the samples (μg/μl) and enable equal loading of the polyacrylamaide gel.

2.4.4 Polyacrylamide Gel Preparation

SDS-PAGE was used comprising:

a) 5% stacking gel (consisting of 7ml de-ionised H_2O, 3ml stacking gel base [consisting of 0.5M TRIS, 0.4% SDS in de-ionised H_2O, pH 6.8], 2ml 30% acrylamide, 20μl 8% bromophenol blue, 24 μl TEMED and 120μl 10% ammonium persulphate)

b) 12.5% running gel x (consisting of 12 ml de-ionised H_2O, 9ml running gel base [consisting of 1.5M TRIS, 0.4% SDS in de-ionised H_20, pH 8.8], 15ml 30% acylamide, 40μl TEMED and 200μl ammonium persulphate)

The SDS in the sample buffer gives the sample proteins a negative charge allowing them to move towards the positive terminal when an electric current is applied. The gel provides a resisting medium for the sample proteins to pass through so that smaller proteins will pass through more easily and therefore faster. In this way the proteins within the sample become separated according to size. A stacking gel is used in order to condense the sample into a thin band when the sample comes across the much higher concentrated running gel. This means that even though the proteins will separate out on the running gel according to size, those proteins with the same size will still be accumulated into a narrow band as that is how they entered the running gel.

A total protein quantity of 60 µg for each sample was loaded into each well in the stacking gel with a broad range Rainbow marker (10µl) loaded into the first well. The gel was then mounted onto a vertical electrophoresis system containing running buffer (glycine 14.4g/L, SDS 1.0g/L, Tris 3 g/L, 1 litre de-ionised H_20). Running water was passed through the central core of the electrophoresis system in order to dissipate heat built up as a result of the electric current from the gel. Gels were run at 200V for 2-4 hours.

2.4.5 Protein Transfer

After the completion of protein separation by electrophoresis, the stacking gel was trimmed off and the running gel was mounted in a transfer system containing transfer buffer (200ml methanol, 700ml de-ionised H_20, 100ml 10x transfer buffer [glycine 14.4g/L, Tris 3 g/L, 1 litre de-ionised H_20]). Hybond ECL nitrocellulose membrane (Amersham, UK) was cut to the same size as the gel and placed on the gel. The gel and membrane were then placed between 2 pieces of Whatman 4mm paper taking care to remove any air bubbles. Following this, the proteins were transferred to the membrane by passing a current of 140mA overnight (16-20 hours). Adequate transfer could be determined by staining the membrane with Ponceau Red (Sigma Chemicals, Poole, UK).

2.4.6 Immunoblotting

The membranes were then washed in PBS-Tween (Na_2HPO_4 1.6 g/L, NaCl 8.0 g/L, de-ionised H_20 1 litre, Tween 20 1ml, pH 7.2), before being placed in blocking buffer (PBS-

Tween with 5% non-fat Marvel) for 1 hour. Blocking buffer was used to block potentially unfilled sites on the membrane and so prevent non-specific binding of any antibodies in the future. The membrane was then washed 3 times in PBS-Tween before incubating in the primary antibody (PBS-Tween, antibody at 1:1000 dilution, 5% BSA) for 2 hours. After washing again 3 times in PBS-Tween, the secondary anti-rabbit antibody (PBS-Tween, secondary antibody 1:1000 dilution, % non-fat Marvel) was placed on the membrane. After washing for a final 3 times, ECL chemi-luminescence reagent was added to the membrane and agitated for 1-2 minutes. Membrane was exposed onto Kodak AR film and developed using Kodak developer and fixer.

2.4.7 Quantification of Bands

The developed films were scanned on using a flatbed document scanner and the digital image produced was analysed using National Institute of Health (NIH) Image version 1.63. Using the supplied 'Gel Plotting Macro' the density of bands was determined employing the grey scale technique.

The loading of the gels was corrected for by measuring each well for β-actin or the total protein of the phosphorylated protein probed for.

2.5 H9c2 Culture

H9c2 culture was provided by the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) catalogue number D-5671, supplemented with 10% v/v foetal calf serum (cat no N-4637), 1% v/v glutamine (cat no G-7513) and 1% v/v penicillin +streptomycin (cat no P-4333).

H9c2 cells were cultured at 37°C and 5% CO_2 (incubator CO28IR, New Brunswick Scientific, USA) and passaged when 80% confluent.

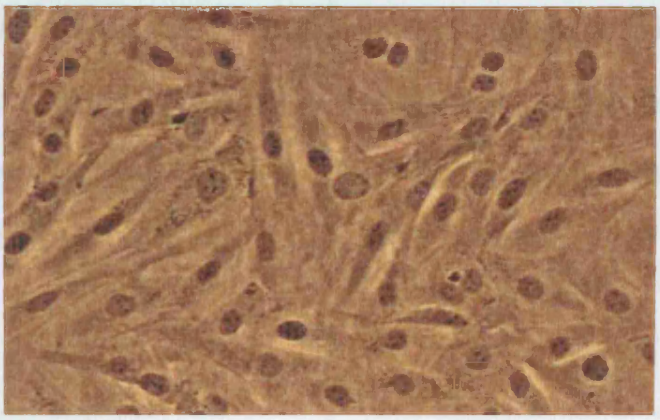


Figure 2. 6, H9c2 cells in culture

2.5.1 Hypoxia/Re-oxygenation

To mimic hypoxia and re-oxygenation a hypoxic chamber was used, see figure 2.7. The chamber consisted of perspex walls and lid to form an air-tight container, heating pads to maintain the temperature at 37°C and an inflow and outflow for inert gas (95% argon, 5% CO_2) to flush out the air. At the bottom of the chamber a dish of sodium dithionite was used to scavenge any oxygen that may linger.

To mimic the physiological conditions of ischaemia the D-5030 medium (Sigma-Aldrich) was used. The main difference between this medium and that used to culture the cells is the presence of 2.2 g/l sodium bicarbonate and 14 mM KCl. The hypoxic medium was supplemented with lactate acid to a final concentration of 20 μM and 1% v/v penicillin-streptomycin, as mentioned above. The pH was measured and found to be 6.5.

In order to induce hypoxia the cell plates were placed in the chamber, which was subsequently sealed. The air in the chamber was removed by vacuum pump and replaced with a 95% argon, 5% CO_2 gas mix. Re-oxygenation was performed by replacing the hypoxic medium with the original culture medium. Any drugs to be administered at re-oxygenation were pre-mixed into the culture medium. Normoxic samples were kept in culture medium. Any washes and medium changes in the hypoxia/reoxygenation groups were matched in the normoxic group.

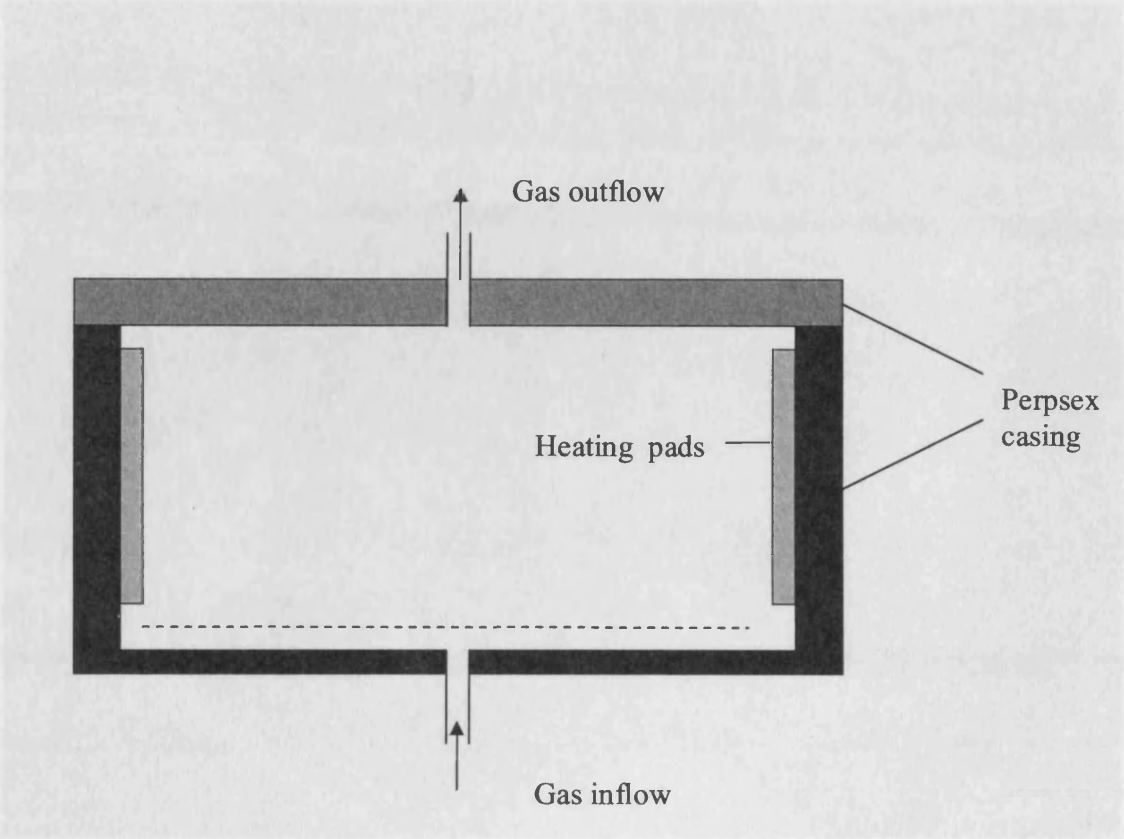


Figure 2. 7, Hypoxic chamber

2.5.2 Flow Cytometry

Flow cytometry is an experimental method whereby cells are passed through a laser and fluorescent markers on or in the cells become excited in a manner that makes it possible to measure the subsequent emmission. In addition, the relative size and density of the cells can be determined by the forward and side scatter of the laser as the cells pass through the beam.

For this purpose, a Partec flow cytometer (Partec, Munster, Germany) with an argon laser emitting light at 488 nm was used.

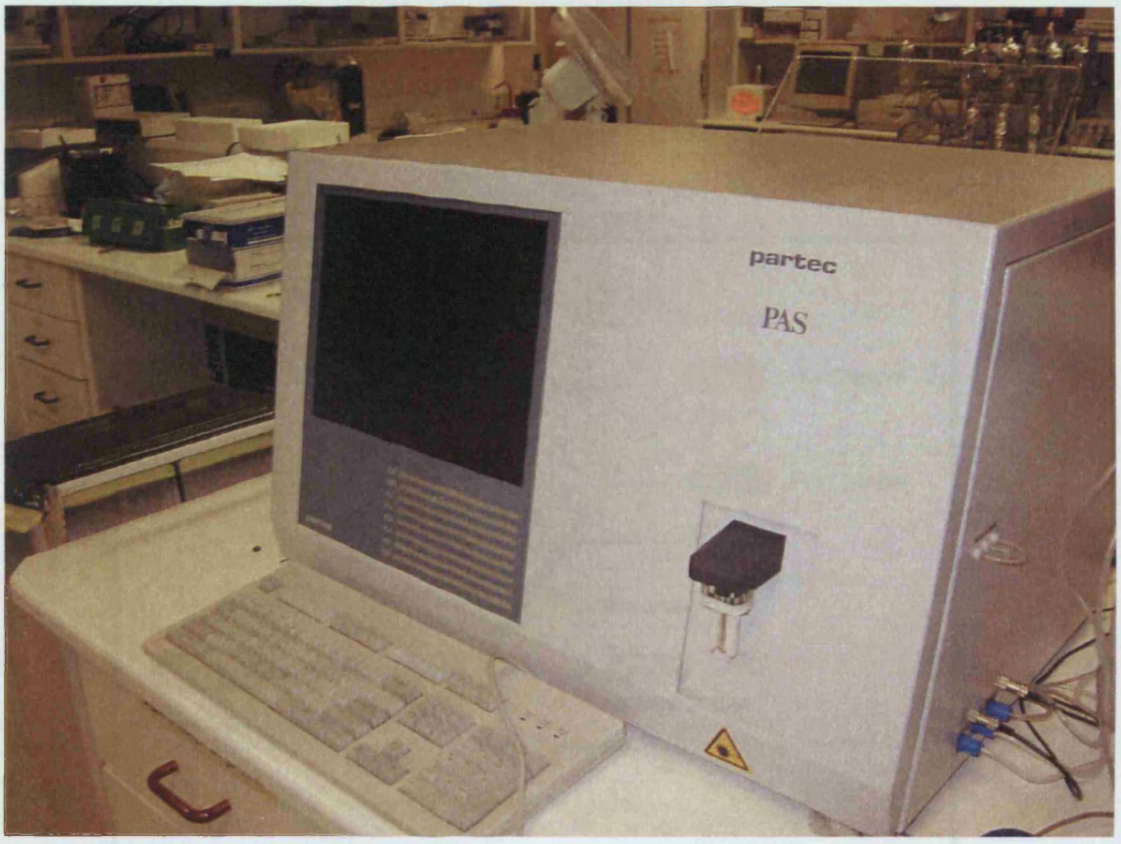


Figure 2. 8, Flow cytometer

In the present study annexin v was used as a marker of apoptosis – and Propidium Iodide (PI) as a marker of necrosis. Annexin v belongs to a family of coagulants known as annexins. It has been shown that annexin binds to phosphatidylserine (PS), which becomes externalised in the early stages of apoptosis. By conjugating the fluorescent marker Fluorescein Isothiocyanate (FITC) to annexin v it is possible to measure the externalisation of PS on the cell membrane and characterise that cell as undergoing apoptosis[138-140].

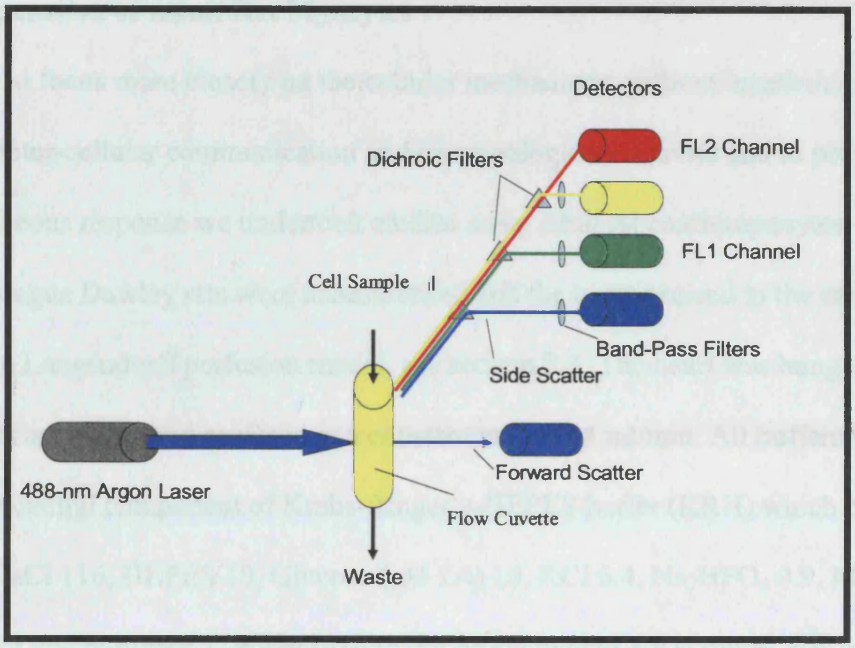


Figure 2. 9, Representation of the mechanics of flow cytometry – Light is emitted by an argon laser and excites the fluorescent probe in the cell passing through the flow cuvette. The light emitted from the cells passes through dichroic filters to the appropriate detector, green to FL1 (annexin v) and red to FL2 (PI).

Similarly, PI enters the cell when the membrane is ruptured making it a useful marker of necrosis. The fact that FITC emits green light when excited and PI red enables the two markers to be used in combination[140].

H9c2 myoblasts that have undergone the experimental protocol are trypsinised (Clonetics, New Jersey, USA) and then incubated for 10 minutes with annexin v (2 µl/ml) in annexin buffer consisting of HEPES (10 mM), NaCl (150 mM), KCl (5 mM) $MgCl_2$ (1 mM) and $CaCl_2$, pH 7.4, at 37°C in the dark. Cells were spun and washed in

phosphate buffered saline (Gibco, Paisley, UK). Just prior to analysis by flow cytometry, PI (50 μg/ml) was added to the sample and mixed.

2.6 Preparation of Adult Rat Myocytes

In order to focus more closely on the cellular mechanisms without interfering with factors such as inter-cellular communication and immunological response and to provide a more homogeneous response we undertook studies using adult rat cardiomyocytes.

Male Sprague Dawley rats were anaesthetised and the heart excised in the same manner as for the Langendorff perfusion model, see section 2.3. The heart was hung on a perfusion apparatus and perfused at a constant rate of 14 ml/min. All buffers were made from the central component of Krebs-Ringer's-HEPES buffer (KRH) which consisted of (mM): NaCl 116, HEPES 10, Glucose (pH 7.4) 10, KCl 5.4, Na_2HPO_4 0.9, $MgSO_4$ 0.4, taurine 20 and pyruvate 5. Water used in the production of KRH buffer was autoclaved and KRH buffer was filter sterilised prior to use.

All buffers were bubbled with 100% O_2 and maintained at 37°C by a heated water jacket. In addition a heart perfusion chamber that acts as a bubble trap just prior to the cannula was also heated by a water jacket.

The heart was initially perfused with buffer 1 consisting of KRH supplemented with 1 mg/ml BSA for 5 minutes. The heart was then perfused with buffer 2 consisting of KRH buffer supplemented with 0.75 mg/ml Type II collagenase and 25 μM $CaCl_2$ for 10 minutes. Finally the heart was perfused with buffer 3 consisting of KRH buffer and supplemented with 50 μM $CaCl_2$ for 5 minutes. At the end of this period the ventricles were excised from the atria and roughly chopped in a Petri dish containing buffer 2. The

mixture was transferred to a 50ml Falcon tube that was gently agitated at 37°C and bubbled with 100% O_2 for 10 minutes. After the first digestion any loose material, usually comprising of dead cells as a result of physical injury from the scissors, was removed and discarded. The remaining material was covered in the collagenase buffer 2 and agitated again for 20 minutes. This was repeated until such time that the myocytes appeared to be at least 70% viable by light microscope. At this point the cells were pipetted off and washed twice in Phosphate Buffered Saline (PBS) and spun at 300g for 2 minutes to remove extraneous fluid. Cells were then resuspended in M-199 medium and 500 µl of the suspension seeded onto coverslips that had had 200 µl of 1mg/ml laminin coated and dried on them. The coverslips were incubated at 37°C with 5% CO_2 (incubator CO28IR, New Brunswick Scientific, USA) to allow the cells to stick to the laminin. At this point 2 ml M-199 were added per well/coverslip.

2.6.1 Confocal Microscopy

Confocal microscopy was performed in order to study the importance of mitochondrial permeability transition. This research was undertaken and conducted in the Mitochondrial Biology laboratories of Professor Michael Duchen using a Zeiss 510 CLSM.

The microscope was set up using a henna laser that was filtered to 543nm with TMRM fluorescence measured at 585 nm by using a long pass filter. Cells were visualised using a 40x oil immersion objective.

The coverslip containing the myocytes was incubated for 15 minutes in 1 µl/ml TMRM and then placed in a custom-made chamber that was inserted onto the objective.

Once suitably healthy cells, as asserted by morphology, had been identified for use within the experiment the cell was scanned by the laser with TMRM emission measured. The timings to the initiation of the mitochondrial wave of membrane depolarisation were measured.

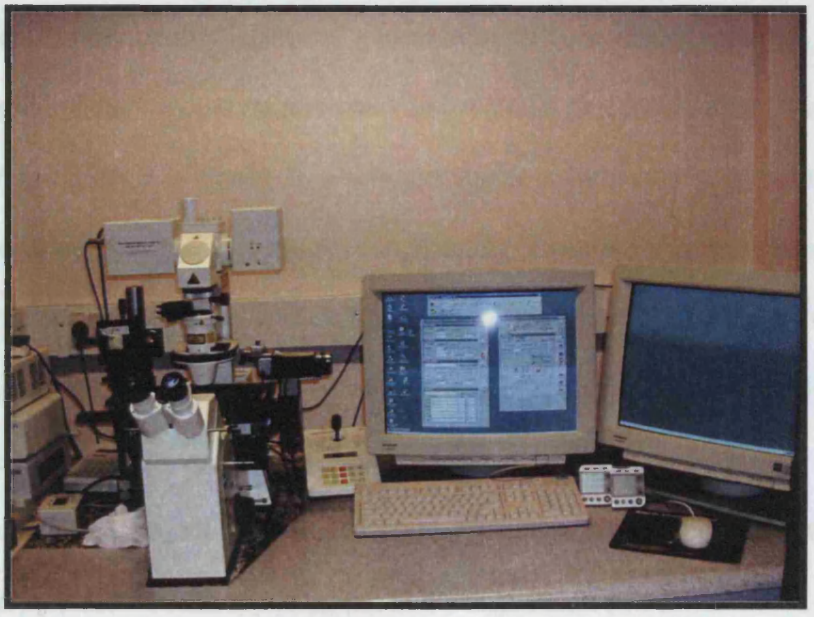


Figure 2. 10, Confocal microscope

2.7 Statistics

Data were statistically analysed by one-way ANOVA. Where a significant F-value was obtained, the Fishers protected least significant difference (PLSD) post hoc test was applied for between group comparisons. All statistics were performed using Statview 4.5 (Abacus Concepts Inc., Berkley, California, USA.). A p-value of <0.05 was considered to be statistically significant. Results are presented as means ± SEM.

Chapter 3 - Erythropoietin Mimics Preconditioning

3.1 Hypothesis, Aims and Protocol

In chapter 1 the phenomenon of preconditioning (brief periods of sub-lethal ischaemia leading to protection against lethal ischaemia) was described. The use of pharmacological agents to mimic this 'classical' preconditioning was also mentioned. Section 1.5.5 explained how recent research had discovered a preconditioning-like phenomenon for EPO in models of stroke. In a similar manner to ischaemic preconditioning (IPC), successful pharmacological preconditioning involves the activation of certain protein kinases such as Akt and PKC. These are kinases that EPO is known to activate [126]. It is reasonable to hypothesise, therefore, that EPO can itself pharmacologically precondition the myocardium via these protein kinases

The aims of the studies included in this chapter were therefore to determine whether EPO can precondition the myocardium in a Langendorff perfusion model and to examine whether the same signal pathways are activated in EPO-mediated protection as that seen in the setting of preconditioning.

3.2 Experimental Protocols

All hearts underwent the standard protocol of 35 minutes ischaemia (index ischaemia) and 2 hours reperfusion, see section 2.3. The following protocols were used:

1. Control – no drug administration, but a stabilisation period of 30 minutes (n=3)

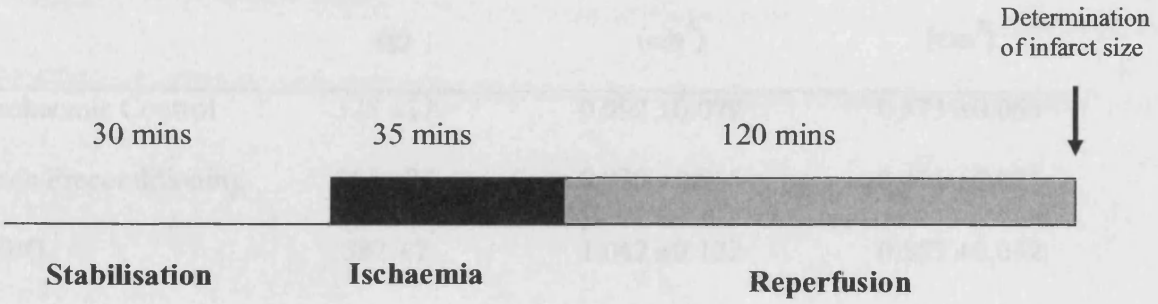
2. Ischaemic Preconditioning – 2 cycles of 5 minutes ischaemia followed by 10 minutes reperfusion prior to index ischaemia (n=3)

3. EPO – administered at a dose of 50 ng/ml for 10 minutes followed by a 10 minute washout prior to entering the index ischaemia (n=5)

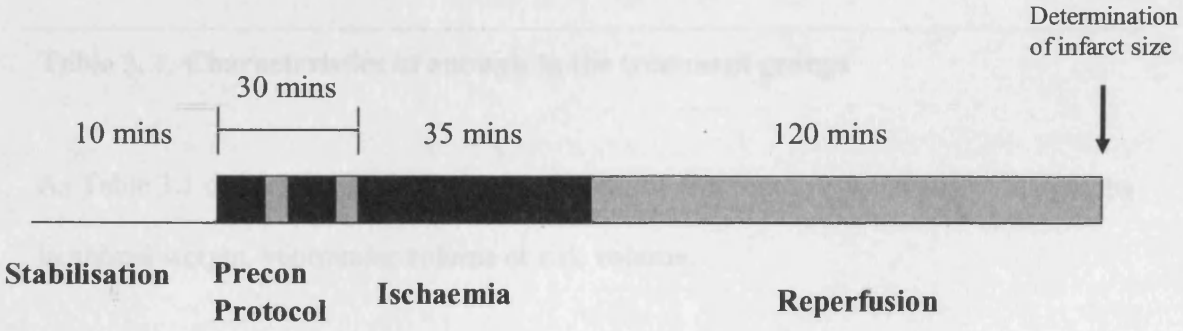
4. Wortmannin – this PI3K inhibitor[141] was administered at a dose of 200 nM either on its own (n=3) or in combination with EPO (n=5)

5. U0126 – this ERK 1/2 inhibitor was administered at a dose of 10 μM either on its own (n=3) or in combination with EPO (n=6)

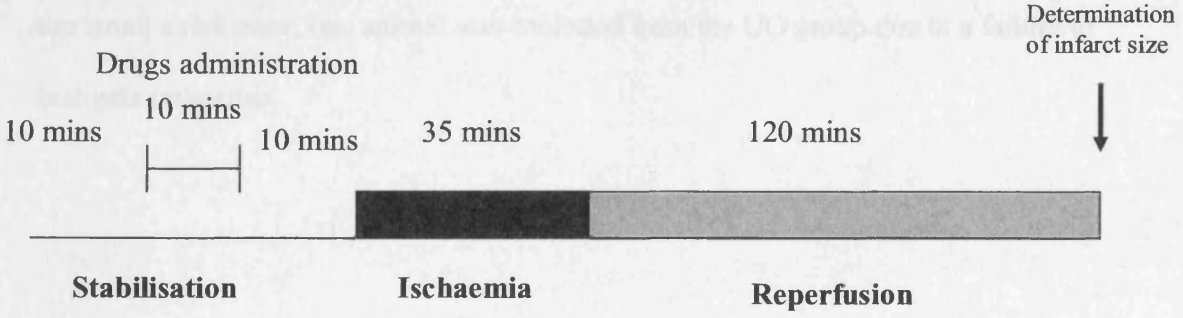
Ischaemic Control



Ischaemic Preconditioning



Pharmacological Preconditioning



3.3 Results

Groups	Body Weight (g)	Ventricle Volume (cm^3)	Risk Volume (cm^3)
Ischaemic Control	375 ±12	0.992 ±0.079	0.573 ±0.055
Isch Preconditioning	364 ±22	0.939 ±0.065	0.471 ±0.028
EPO	382 ±7	1.042 ±0.122	0.557 ±0.052
EPO + Wort	373 ±12	1.059±0.027	0.587 ±0.044
EPO + U0	379 ±2	1.057 ±0.049	0.542 ±0.050
Wort	350 ±24	0.959 ±0.065	0.569 ±0.060
U0	359 ±14	1.004 ±0.060	0.501 ±0.033

Table 3. 1, Characteristics of animals in the treatment groups

As Table 3.1 demonstrates, there was no statistical difference between any of the groups in animal weight, ventricular volume or risk volume.

Exclusions

One animal from the ischaemic preconditioning group was excluded due to too large a risk zone, two animals were excluded from the EPO group for either having too large or too small a risk zone, one animal was excluded from the UO group due to a failure to instigate ischaemia.

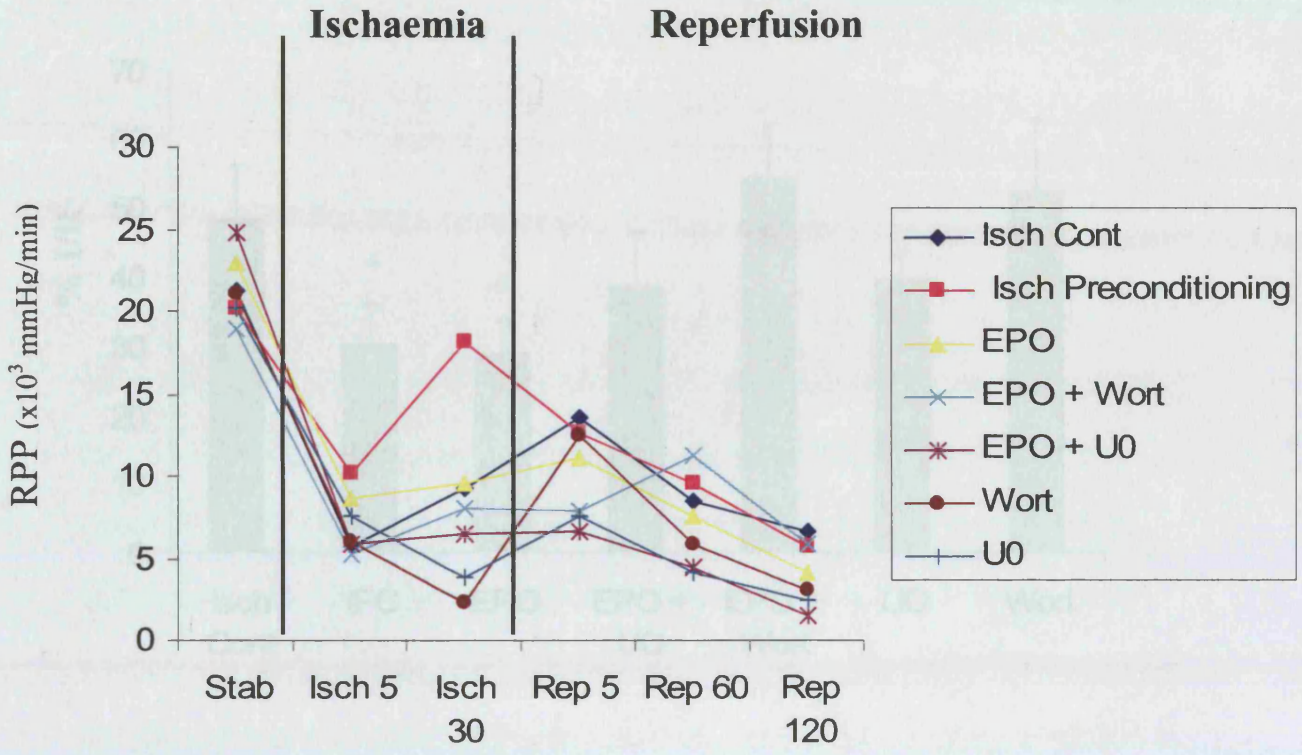


Figure 3. 1, Representation of the change in Rate-Pressure Product (RPP) throughout the experimental protocol from stabilisation (Stab) through ischaemia (Isch) and into reperfusion (Rep)

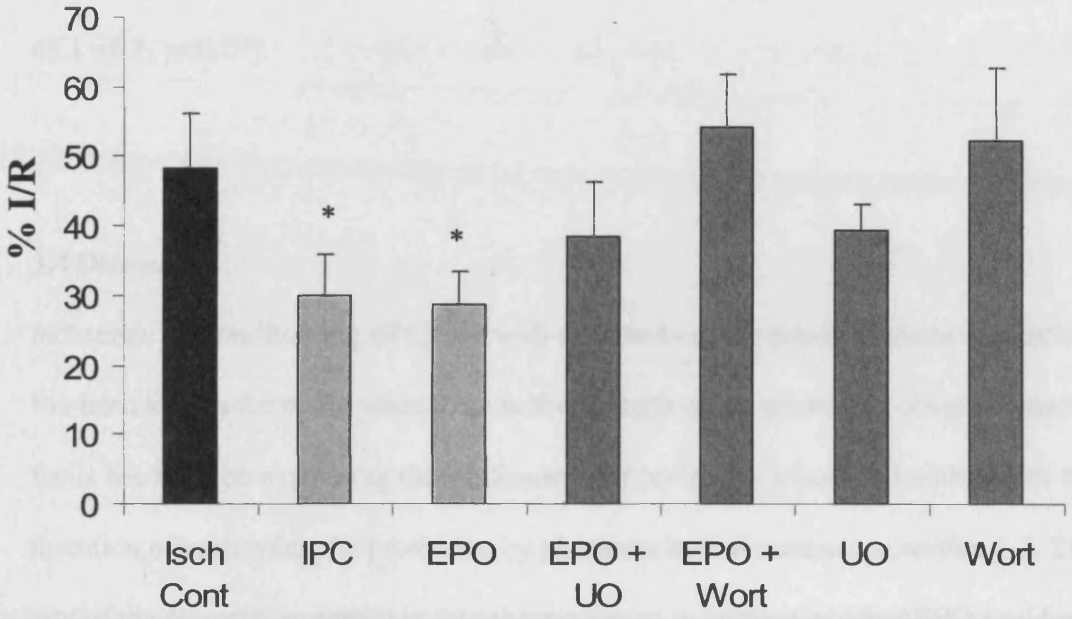


Figure 3. 2, The effect of ischaemic preconditioning (IPC), and of a 50 ng/ml dose of EPO when administered as a preconditioning mimetic solely, or with the PI3K inhibitor wortmannin (Wort) or the ERK 1/2 inhibitor UO126 (UO). The effect of Wort and UO alone on infarction is also presented. Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *$p<0.05$ vs control.

Fig 3.2 describes the significant reduction in infarct size when EPO is administered prior to the index ischaemia compared to control (%I/R 28.6 ±4.8 v 48.1 ±8.1, $p<0.05$). This reduction in infarct size is lost when EPO is perfused in combination with wortmannin (%I/R 54.2 ±7.6 v 28.6 ±4.8, $p<0.05$). U0126 did not significantly reduce the infarct-limiting effects of EPO when co-perfused (%I/R 38.4 ±8.1 v 28.6 ±4.8). Neither wortmannin nor U0126 administered solely significantly altered infarct size compared to control (%I/R 52.2 ±10.6; 39.5 ±3.7 v 48.1 ±8.1, respectively). As expected, ischaemic

preconditioning reduced infarct size significantly compared to control (%I/R 29.8 ±6.3 v 48.1 ±8.1, $p<0.05$).

3.4 Discussion

Ischaemic preconditioning (IPC) is a well-accepted cardioprotective phenomenon that has been known for many years. Due to the strength of the protection observed much focus has been on examining the mechanisms of protection associated with it with the intention of replicating the protection by pharmacological means, see section 1.3. The aim of the research presented in this chapter was to determine whether EPO could mimic IPC when administered prior to the index ischaemia and whether any protection observed is achieved via similar pathways as described for IPC and other successful preconditioning mimetics.

3.4.1 EPO Mimics Preconditioning

EPO-mediated cardioprotection against ischaemia/reperfusion injury has begun to be well characterised in the last few years as discussed in chapter 1. Commonly, the effect has been seen when EPO is administered during the ischaemic period or just prior to commencement of ischaemia. Calvillo et al were the first to describe EPO-mediated protection when they administered the agent pre-ischaemically in an in vivo model of infarction and in adult rat cardiomyocytes[142]. Tramontano et al also demonstrated this cardioprotective effect in a neonatal rat cardiomyocyte model in addition to an in vivo rat open chest model[143]. Parsa et al used adult rat cardiomyocytes and the rat-derived

ventricular myoblast cell line, H9c2, to show that EPO could protect against H_2O_2-induced injury[144]. These studies have been supported by the findings of several other papers examining the effect of EPO in cellular models of hypoxia[142, 144]. Importantly, the phenomenon has not only been seen in cellular models but has been reproduced using both in vitro and in vivo models of ischaemia/reperfusion[121, 145, 146], discussed in detail later on. The effect shown was not merely to delay tissue death to beyond the point of a short period of reperfusion. Moon et al gave EPO in an in vivo rat model at the point of ligation of the coronary artery to induce ischaemia without reperfusion. As an end-point they measured apoptosis 24 hours later and infarct size and ventricle size 8 weeks later[147]. They found a positive effect in all parameters thus showing that the effect was not merely a delay but a long-lasting prevention of cell death. A contradictory study, however, by Hale et al found that the infarct size in a permanent coronary occlusion rat model 6 weeks after commencement of ischaemia was no different in EPO-treated hearts than control[148]. As the authors themselves conceded, however, timing and dosage of EPO seems to be important and their decision to dose daily for the first 7 days may have meant that the dose of EPO was outside the therapeutic window. One study by Kristensen et al also contradicts the data in the literature presented here. They found that EPO had no effect on infarct size when administered prior to ischaemia in an in vivo porcine model of ischaemia/reperfusion[149]. The differences reported by this group may be attributed to the species used in the experiment. The pig model is known to have less collateral flow than the rat [150, 151] and thus may contribute to the difference in the data produced. The pig may also respond differently to human recombinant EPO than the rat. Another major

difference is that the Kristensen study used a dose of 500 U/Kg which is 10-fold less than the dose used habitually within the rat.

All the models mentioned thus far have involved EPO being present up to at least the commencement of index ischaemia/hypoxia. However, one of the main features of preconditioning is the memory effect affording a protected state within the tissue even after the stimulus has been removed. As can be observed from the results presented in this chapter, EPO clearly managed to elicit just such a memory effect within the myocardium since EPO still limited ischaemia/reperfusion-induced injury despite having been washed out. Therefore, the data presented within this chapter are the first to show that EPO can precondition the myocardium. A memory effect of EPO has only been produced in a neuronal setting[152]. Most other studies that have investigated the effects of EPO pre-treatment did not include a wash-out period prior to index ischaemia. Thus it cannot be argued that the protection seen is due to the memory effect characteristic of preconditioning [127, 153].

3.4.2 EPO Mimics Preconditioning through PI3K

From the data presented in figure 3.2 it can quite clearly be seen that EPO-mediated protection is abolished when EPO is co-administered with the PI3K blocker wortmannin[141], suggesting that the protection is PI3K-dependent. The fact that PI3K seems to be involved in the protective effects of EPO should not come as a surprise as it has been found that the method EPO uses to prevent apoptosis in erythroid progenitor cells is by PI3K activation[154, 155]. Indeed, IPC itself has been associated with being PI3K-

dependent[39]. Our data suggest that EPO utilises at least some of the same protein kinase signalling pathways as IPC. Other studies regarding the EPO-mediated cardioprotection have likewise implicated PI3K as an important kinase activated by EPO[156]. This includes data produced by Tramontano et al, who found that PI3K was an important factor in EPO-mediated protection against hypoxia in cardiomyocytes[143]. In addition, Hanlon et al[145] used the alternative PI3K blocker, LY294002, as well as wortmannin, to demonstrate that EPO-mediated protection was PI3K-dependent in a similar rat Langendorff perfusion model. This model involved a protocol consisting of 20 minutes of global ischaemia and 40 minutes of reperfusion. Interestingly, they found that the co-administration of blockers with EPO prior to ischaemia had no effect on the reduced infarct size shown by EPO administered solely [145]. However, when the blockers were given at reperfusion, protection was abolished. This fact seems to suggest that the protective effect of EPO is effective against reperfusion-induced injury. Interestingly, Hausenloy et al demonstrated that IPC could be inhibited when LY294002 was administered not only prior to ischaemia[44] but, more importantly, when it was given at reperfusion. Against this argument is the data published by Moon et al, as mentioned in section 3.4.1, which indicated that EPO could limit injury in a model that did not include reperfusion[147]. Hanlon et al go on to suggest that PKCs, and in particular the epsilon isoform of PKC, are important in mediating the protection afforded by EPO as shown by the movement of PKC epsilon to the intercalated disc subsequent to EPO administration. The importance of PKC was shown by the fact that the PKC inhibitor chelerythrine abolished EPO-mediated protection in their model[145]. Both the movement of PKC epsilon

and the attenuation of protection by chelerythrine applies to IPC-mediated protection [157-162], suggesting further similarities in protective pathways between the two stimuli.

3.4.3 EPO-induced Preconditioning is not ERK 1/2-dependent

The data presented in this chapter appears to indicate that the ERK inhibitor, U0126, failed to significantly abolish the protection seen by pre-ischaemic administration of EPO. It should be noted, however, that the infarct size for EPO+U0 and U0 alone were similar making the result ambiguous. Interestingly, ERK 1/2 has not been implicated in mediating the cardioprotection seen as a result of the effects of IPC[39]. The same can be said for EPO, as shown by Hanlon et al[145], although Rafiee et al managed to inhibit the protection provided by EPO in the rabbit infant heart with the ERK 1/2 inhibitor PD98059[127]. The difference in response could be attributed to the different inhibitor, the different species or different age of the animal used. This absence of ERK involvement in preconditioning is in contrast to the importance attributed to ERK in EPO-mediated protection at reperfusion, see chapter 4. The ERK-independent nature of the protective effects of a pre-treatment of EPO may be specific to the heart. In this regard Kilic et al have shown that in the brain ERK does seem to be involved in protection induced prior to and during ischaemia[163]. There are, as yet, no further studies that have examined the role of ERK within EPO-mediated protection. Thus, these findings have yet to be confirmed by additional studies. It should be noted that the control U0126 value is very similar to the U0 + EPO group, suggesting that the drug itself may be having an effect. The body of studies using U0126 and examining the role of ERK 1/2 at preconditioning suggests that the U0126 group result is likely to be an artefact. Therefore, more research needs to be undertaken to clarify the data.

3.4.4 Clinical Implications of EPO Preconditioning

The difficulty in transferring the positive results of preconditioning to the clinic has always been one of the predictability of adverse events in patients. As the window of protection provided by preconditioning stimuli is relatively short at 1-2 hours[24, 164, 165], it requires knowledge of a pending coronary occlusion to be best able to utilise the intervention, in this case EPO. There are instances where the ischaemic period is predictable, such as the elective operation Coronary Artery Bypass Grafts (CABG). In this technique there is scope to use EPO to mimic preconditioning in the heart prior to the operation in order to reduce any damage caused by the procedure. So as to provide a wide-ranging use for the protective qualities of EPO, an alternative setting is required. In this regard, using EPO as an adjunct to reperfusion may provide a more effective clinical use.

Chapter 4 - EPO in Reperfusion Injury

4.1 Introduction and Aims

There has been a significant amount of research surrounding the possible triggers and end-effectors of preconditioning, but the difficulty has always been establishing this into clinical practice. By its very nature, to use preconditioning effectively would require the ability to predict a sustained ischaemic occurrence which would lead to an infarction, see chapter 3. Certainly in emergency cases the ischaemic period has already commenced and so the main treatment required is to remove the obstructive thrombus causing the myocardial ischaemia and institute a reperfusion of the tissue as quickly as possible. While this treatment is clearly necessary, paradoxically this reperfusion can cause injury to the tissue over and above that caused by ischaemia, a phenomenon known as 'reperfusion-induced injury' [45]. Research has shown that a variety of agents can limit cell death when given at reperfusion, see section 1.4, thus confirming the phenomenon and opening up potential therapeutic possibilities.

As described in chapter 1, reperfusion injury is a concept that has profound implications in the treatment of patients in both an elective and emergency scenario. As the use of EPO has been demonstrated to limit cell death in a number of settings and is able to activate mechanisms proven to be effective against reperfusion injury, it is hypothesised that EPO could reduce myocardial injury when administered at the point of reperfusion.

4.1.1 Mechanisms of Intervention

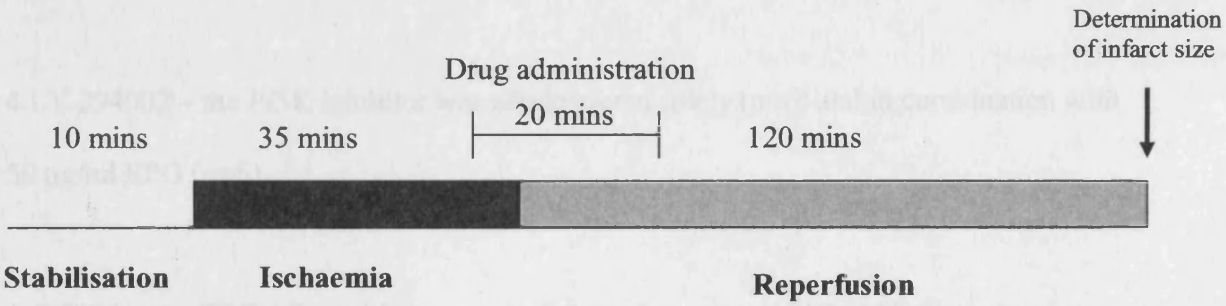
Recently it has been hypothesised that reperfusion-induced injury is triggered by the opening of the mPTP. This causes cytochrome c release, leading to initiation of apoptosis[166-168] and ATP depletion resulting in necrotic cell death [169, 170]. Early research into reperfusion injury suggested that free radical scavengers could reduce reperfusion-induced injury [46, 171, 172], implying that oxidative stress was a main factor in reperfusion injury. However, results of this type could not be replicated in the clinical setting by improving the outcome of patients despite the biochemical markers of their stress being improved [173-175]. Studies elucidating the role of ROS within the cell have demonstrated that lower concentrations of ROS can have a beneficial effect by activating pro-survival kinase pathways such as the ERK pathway via G proteins.[176-178]

Research focus has shifted to the protein kinase survival pathways, highlighting in particular a beneficial pathway at reperfusion, the so-called RISK (Reperfusion Injury Salvage Kinase) pathway [61]. EPO is known to activate certain elements of this pathway such as ERK 1/2 [126] and Akt. It is therefore possible to hypothesise not only that EPO limits reperfusion-induced injury, but also that it does so utilising the RISK pathway.

4.2 Experimental Protocol

All hearts were prepared and subject to a standard ischaemia/reperfusion protocol using the Langendorff apparatus described in chapter 2. Due to the novel nature of the work, a dose response curve was produced initially in order to try and determine the most effective dose to study. In choosing the range we were guided by a study by Calvillo et al that used an in vivo dose of 5,000 U/kg, equivalent to 50 ng/ml[142].

EPO was dissolved in Krebs-Heinselet buffer to produce the following concentrations: 0 (n=6), 20 (n=5), 50 (n=5), 100 (n=8), 200 (n=8) ng/ml and perfused 5 minutes prior to reperfusion for a total of 20 minutes as outlined below.



Once the dose had been confirmed, the mechanism of protection was studied using pharmacological inhibitors. The following protocols were used:

1. Control – where no drug was administered (n=7)

2. EPO – administered at a dose of 50ng/ml (n=7)

3. Wortmannin – the PI3K inhibitor was administered at a dose of 200nM both on its own (n=7) and in combination with 50ng/ml EPO (n=5)

4 LY 294002 – the PI3K inhibitor was administered solely (n=6) and in combination with 50 ng/ml EPO (n=6)

5. U0126 – the ERK 1/2 inhibitor was administered at a dose of 10 μM both on its own (n=5) and in combination with 50 ng/ml EPO (n=6)

6. Denatured EPO – EPO sample was boiled at 100°C for 10 minutes in order to change the protein structure and administered at a dose of 50 ng/ml (n=5)

7. L-NAME – the NOS inhibitor was administered at a dose of 100 μM both on its own (n=5) and in combination with 50 ng/ml EPO (n=5)

4.2.1 Western Blotting

In order to determine the phosphorylation status of certain key protein kinases, Western blots were performed as described in chapter 2. Pharmacological inhibitors were also used to establish the effect of eliminating a kinase's activity on survival pathways. The protocols used were as followed:

1. Control – no drug administration (n=6)

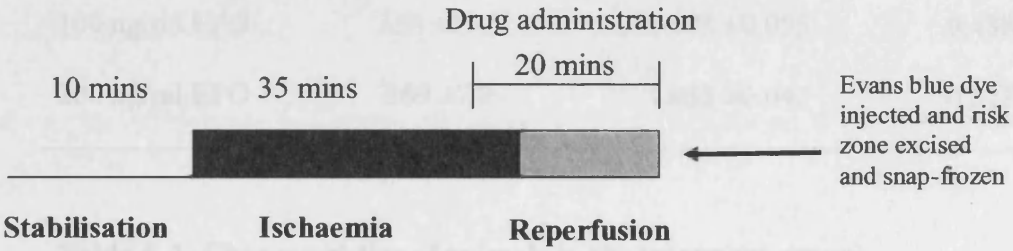
2. EPO – EPO was administered at a dose of 50 ng/ml (n=6)

3. Wortmannin – the PI3K inhibitor was administered at a dose of 200 nM both on its own (n=6) and in combination with 50 ng/ml EPO (n=6)

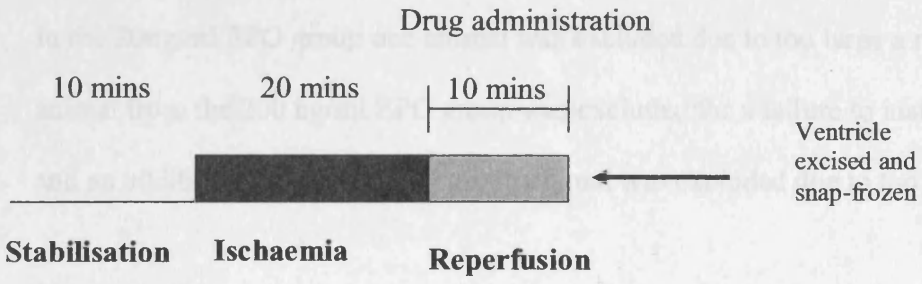
4. U0126 – the ERK 1/2 inhibitor was administered at a dose of 10 μM both on its own (n=6) and in combination with 50 ng/ml EPO (n=6).

Two protocols of ischaemia/reperfusion were used to prepare samples for Western blotting: a) regional ischaemia involved ligating the LAD in exactly the same way as for the infarct model and b) global ischaemia involved stopping all flow to the myocardium:

Regional Ischaemia



Global Ischaemia



The phosphorylation states of Akt and ERK were measured in both protocols whereas the phosphorylated state of eNOS was determined only in the global ischaemia protocol.

4.3 Results

4.3.1 Dose Response

Groups	Body Weight(g)	Muscle Volume (cm^3)	Risk Volume(cm^3)
Ischaemic Control	361 ±6.6	0.979 ±0.075	0.443 ±0.024
20 ng/ml EPO	353 ±22.7	0.965 ±0.103	0.454 ±0.052
50 ng/ml EPO	370 ±12.2	1.074 ±0.150	0.531 ±0.072
100 ng/ml EPO	358 ±15.5	1.008 ±0.055	0.488 ±0.060
200 ng/ml EPO	369 ±7.9	1.033 ±0.040	0.522 ±0.032

Table 4. 1, Characteristics of animals in the treatment groups

Exclusions

In the 20ng/ml EPO group one animal was excluded due to too large a risk zone, one animal from the 200 ng/ml EPO group was excluded for a failure to instigate ischaemia and an additiona 200 ng/ml EPO group animal was excluded due to too large a risk zone.

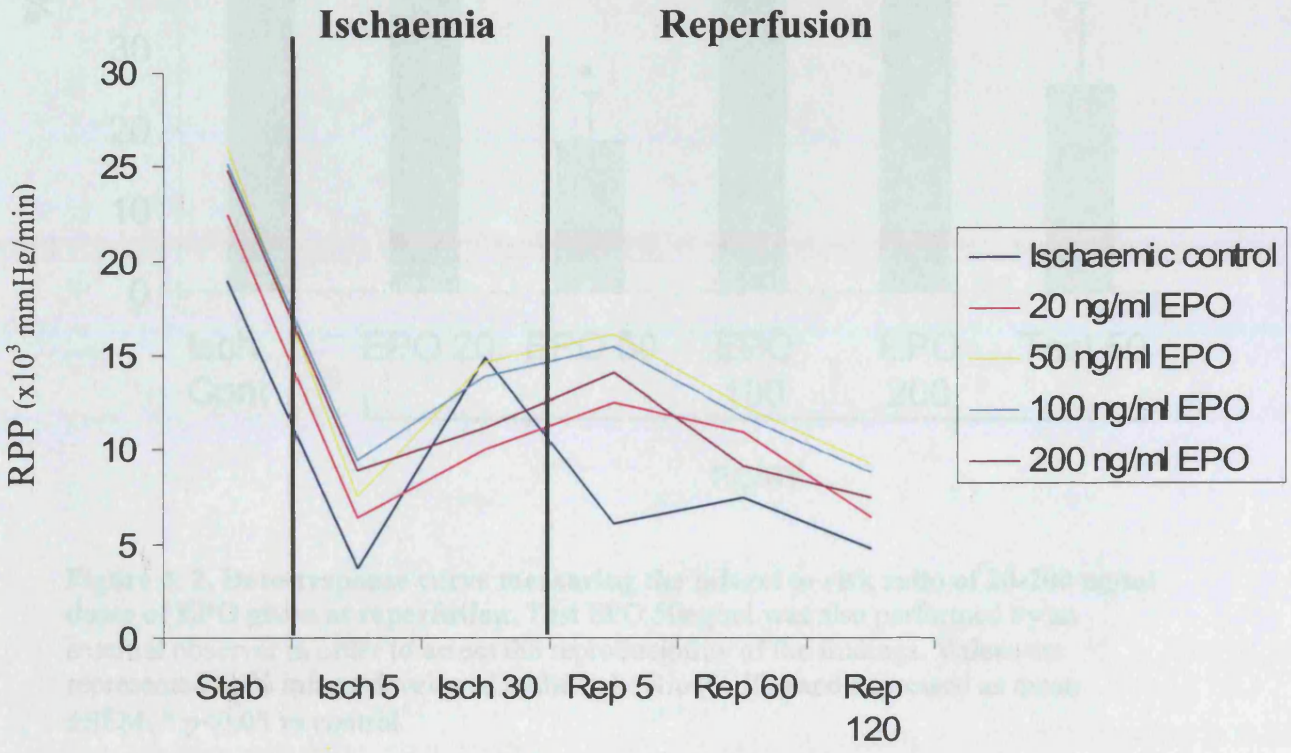


Figure 4. 1 Representation of the change in Rate-Pressure Product (RPP) throughout the experimental protocol from stabilisation (Stab) through ischaemia (Isch) and into reperfusion (Rep)

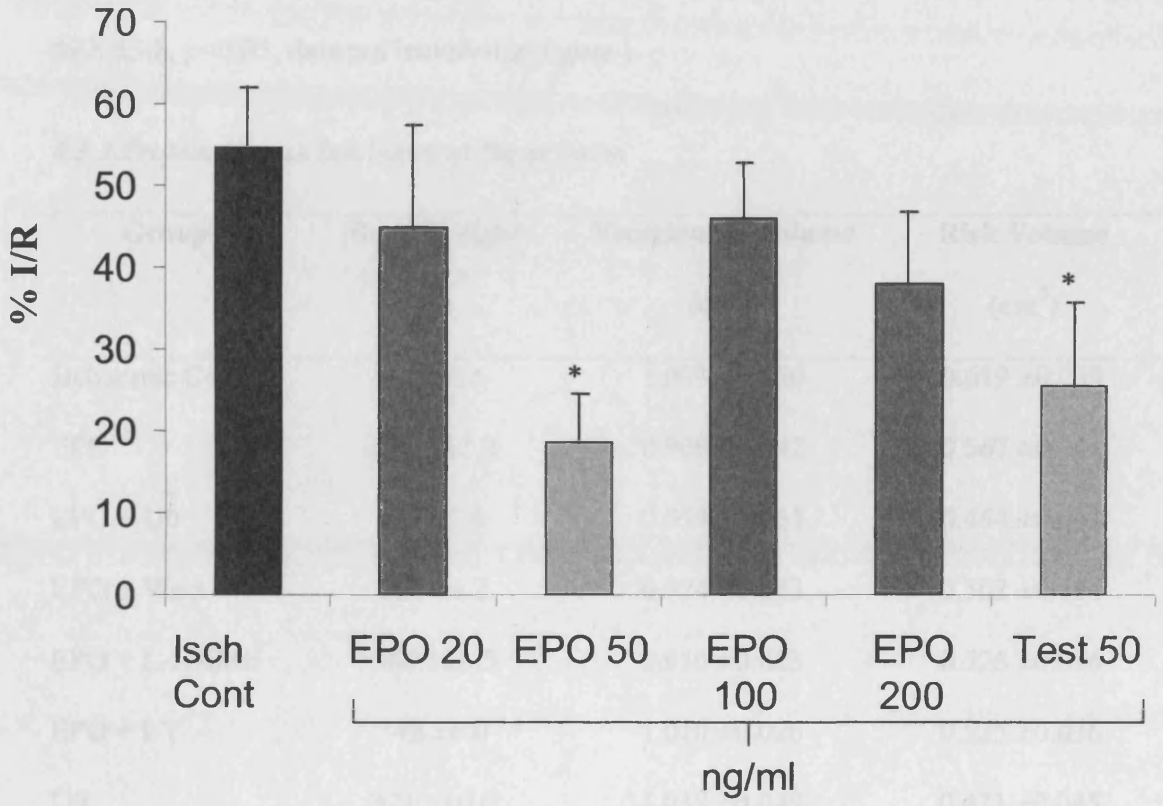


Figure 4. 2, Dose-response curve measuring the infarct to risk ratio of 20-200 ng/ml doses of EPO given at reperfusion. Test EPO 50ng/ml was also performed by an external observer in order to assess the reproducibility of the findings. Values are represented as % infarct developed in the risk ratio (% I/R) and expressed as mean ±SEM. * $p<0.05$ vs control.

As figure 4.2 describes, only the 50 ng/ml dose group significantly reduced infarct size compared to the ischaemic control (%I/R 18.6 ±5.9 v 54.5 ±7.4, $p<0.05$). The doses of 20 ng/ml (%IR 44.8 ±12.5), 100 ng/ml (%IR 46.1 ±6.9) and 200 ng/ml (%IR 37.9 ±9.0) all failed to show significant protection compared to control.

The data obtained by the external observer confirm the findings that the 50 ng/ml EPO induces significant protection compared to external observer's control (IR% 25.5 ±10.1 v 49.8 ±3.8, $p<0.05$, data not included in figure).

4.3.2 Protein Kinase Inhibitors at Reperfusion

Groups	Body Weight (g)	Ventricular Volume (cm^3)	Risk Volume (cm^3)
Ischaemic Control	364 ±8.6	1.093 ±0.050	0.619 ±0.039
EPO	370 ±12.2	0.908 ±0.042	0.567 ±0.041
EPO + U0	313 ±7.9	0.959 ±0.051	0.464 ±0.031
EPO + Wort	317 ±4.2	0.974 ±0.043	0.502 ±0.034
EPO + L-NAME	368 ±16.5	0.950 ±0.023	0.526 ±0.026
EPO + LY	348 ±6.0	1.010 ±0.026	0.525 ±0.036
U0	321 ±10.0	1.038 ±0.049	0.421 ±0.045
Wort	333 ±9.4	1.049 ±0.049	0.583 ±0.040
L-NAME	368 ±16.5	0.969 ±0.042	0.523 ±0.034
Den EPO	342 ±12.5	0.953 ±0.050	0.527 ±0.019

Table 4. 2, Characteristics of animals in the treatment groups

Exclusions

One animal from the EPO + Wort group was excluded due to too large a risk zone, one animal from the EPO + U0 group was excluded due to an infarct size of less than 5% and another EPO + U0 animal was excluded as the perfusion pressure failed mid-experiment.

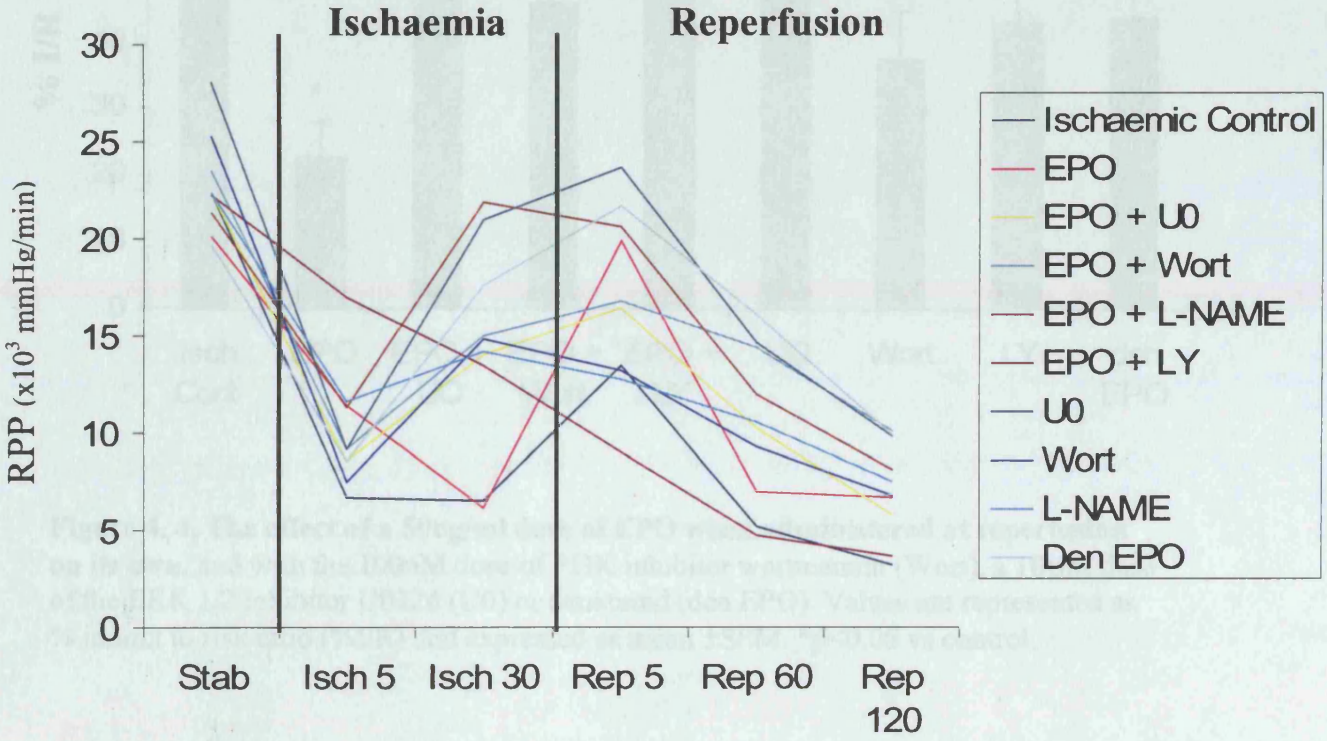


Figure 4. 3, Representation of the change in Rate-Pressure Product (RPP) throughout the reperfusion experimental protocol from stabilisation (Stab) through ischaemia (Isch) and into reperfusion (Rep)

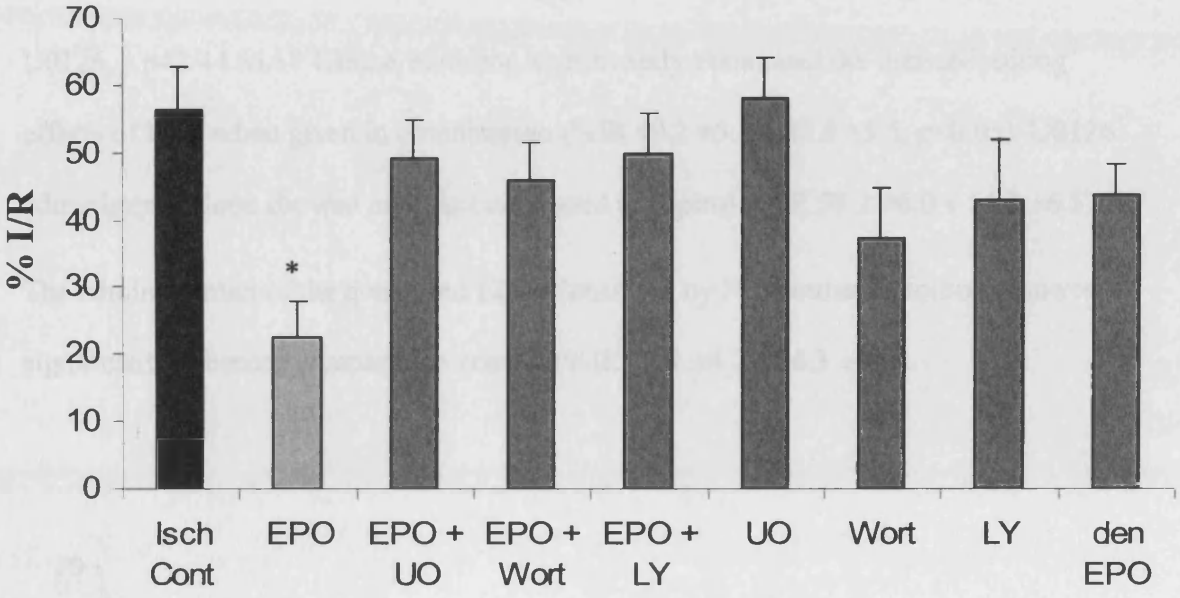


Figure 4. 4, The effect of a 50ng/ml dose of EPO when administered at reperfusion on its own, and with the 100nM dose of PI3K inhibitor wortmannin (Wort), a 10μM dose of the ERK 1/2 inhibitor U0126 (U0) or denatured (den EPO). Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *$p<0.05$ vs control.

As with the dose response curve, a 50 ng/ml dose of EPO significantly reduced the infarct to risk percentage compared to control (%I/R 22.5 ±5.5 v 56.3 ±6.5, $p<0.05$). The PI3K inhibitor, wortmannin, significantly abolished the infarct-limiting properties of EPO when given in combination (%IR 46.1 ±5.4 v 22.5 ±5.5). Interestingly, wortmannin administered alone showed a significantly beneficial effect compared to control (%IR 37.4 ±7.4 v 56.3 ±6.5, $p<0.05$).

Likewise, LY294002, a PI3K inhibitor, abrogated the beneficial effect of EPO compared to EPO administered alone (%IR 49.9 ±6.1 v 22.5 ±5.5, $p<0.05$). LY294002 administered on its own showed no effect compared to control (%IR 43.0 ±9.0 v 56.3 ±6.5).

U0126, a p42/44 MAP Kinase inhibitor, significantly eliminated the infarct-limiting effects of EPO when given in combination (%IR 49.2 ±5.6 v 22.5 ±5.5, $p<0.05$). U0126 administered alone showed no effect compared to control (%IR 58.2 ±6.0 v 56.3 ±6.5).

The administration of the denatured EPO (denatured by 10 minutes of boiling) showed no significant difference compared to control (%IR 43.9 ±4.7 v 56.3 ±6.5).

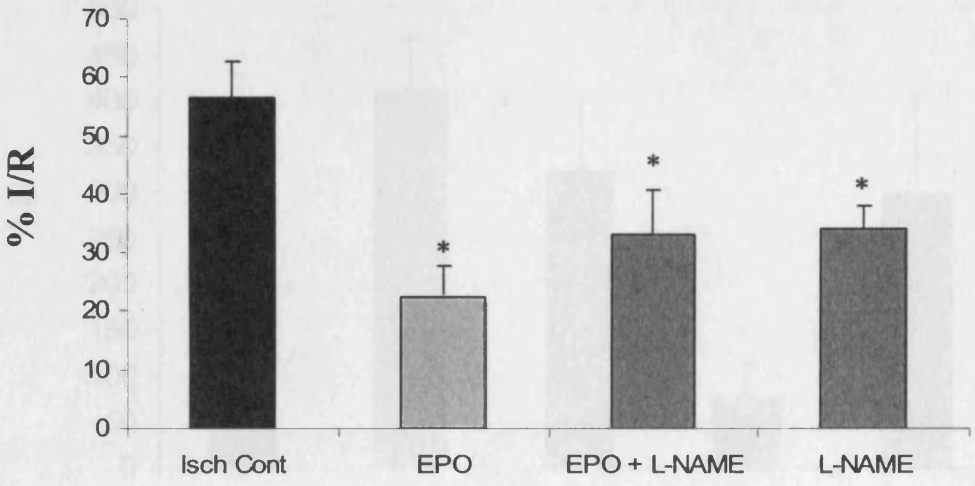


Figure 4. 5, The effect of a 50ng/ml dose of EPO when administered at reperfusion solely, and with a 10μM dose of NOS inhibitor L-NAME. Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *$p<0.05$ vs control.

EPO+L-NAME demonstrated a significantly smaller infarct size compared to control (%IR 33.1 ±7.5 v 56.3 ±6.5, $p<0.05$). L-NAME administered on its own also showed a significant decrease compared to control (%IR 34.1 ±3.8 v 56.3 ±6.5).

4.3.3 Western Blotting

4.3.3.1 Regional Ischaemia

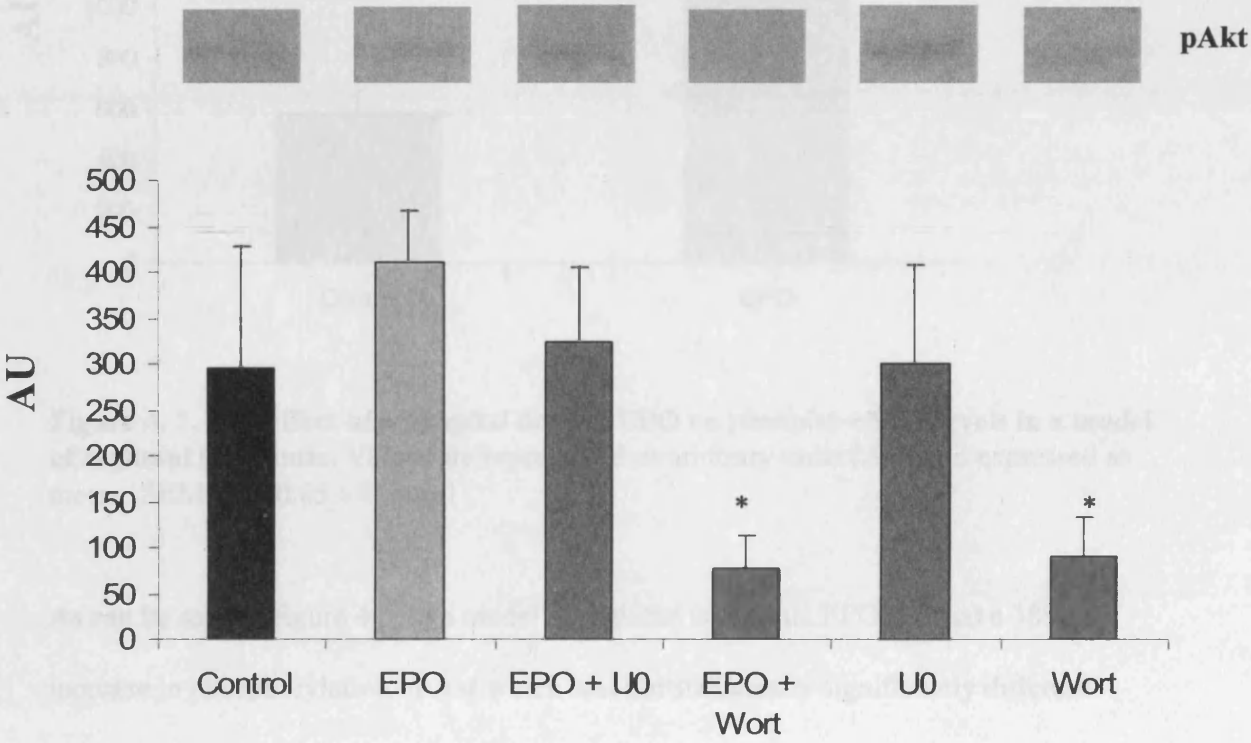


Figure 4. 6, The effect of a 50ng/ml dose of EPO on phospho-Akt levels in a model of regional ischaemia with or without the Erk inhibtor U0126 (U0) and the PI3K inhibitor wortmannin (Wort).Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ vs Saline Naive

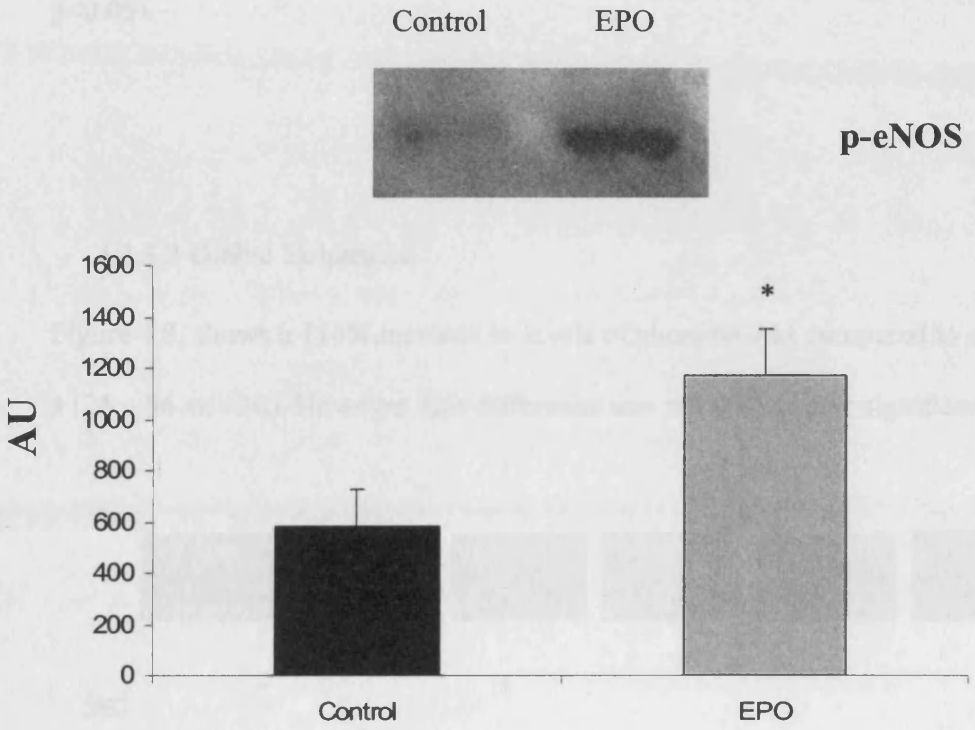


Figure 4. 7, The effect of a 50ng/ml dose of EPO on phospho-eNOS levels in a model of regional ischaemia. Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ v Control

As can be seen in figure 4.6, in a model of regional ischaemia EPO showed a 38% increase in phosphorylation of Akt which was not statistically significantly different compared to control (409 au ±55 v 296 au ±131). The groups that included U0126 did not show any significant change compared to control (EPO + U0, 324 au ±79; U0 300 au ±108). Groups that were treated with wortmannin showed a 75% (EPO + Wortmannin, 76 au ±37) and 70% (Wortmannin, 91 au ±42) reduction in phospho-Akt levels that was statistically significantly different from control (296 ±131, $p<0.05$).

Figure 4.7 shows a 100% increase in phospho-eNOS levels for the EPO treated group compared to control that was statistically significant (1168 au ±181 v 582 au ±147, $p<0.05$).

4.3.3.2 Global Ischaemia

Figure 4.8, shows a 110% increase in levels of phospho-Akt compared to control (206 au ±126 v 96 au ±30). However, this difference was not statistically significantly different.

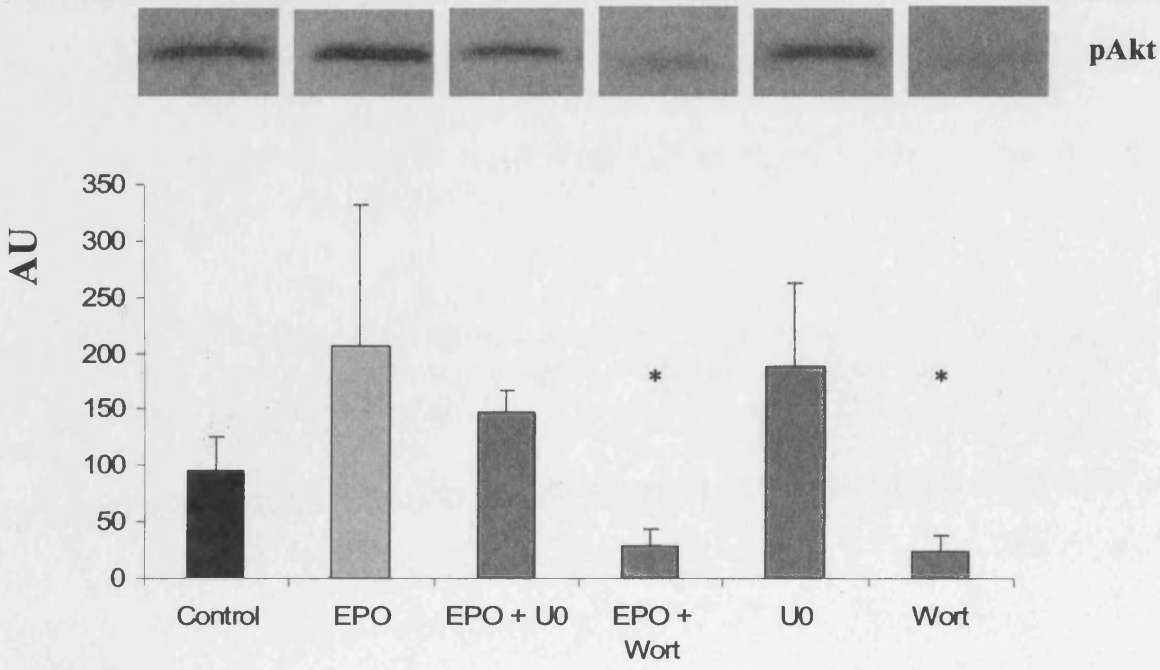


Figure 4. 8, The effect of a 50ng/ml dose of EPO on phospho-Akt levels in a model of global ischaemia. Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ vs Control

Groups treated with U0126 also did not show any statistical difference compared to control (EPO + U0, 148 au ±19; U0, 188 au ±73).

Figure 4.9 shows that hearts treated solely with EPO showed a non-significant increase compared to control of 40% in phospho-ERK1 levels (267 au ±128 v 190 au ±45) and 60% in phospho-ERK2 levels (285 au ±150 v 177 ±36). Hearts treated with EPO plus wortmannin did not vary significantly from either ERK1 (194 au ±35) or ERK2 (172 au ±30) phosphorylation levels compared to control. The same can be said of the groups treated solely with wortmannin for (ERK1, 260 au ±47; ERK2 au ±47). Hearts treated with U0126 + EPO showed a significant 98% drop in ERK1 phosphorylation levels (5 au ±5) as well as a total absence of a signal for ERK2 (0 au ±0).

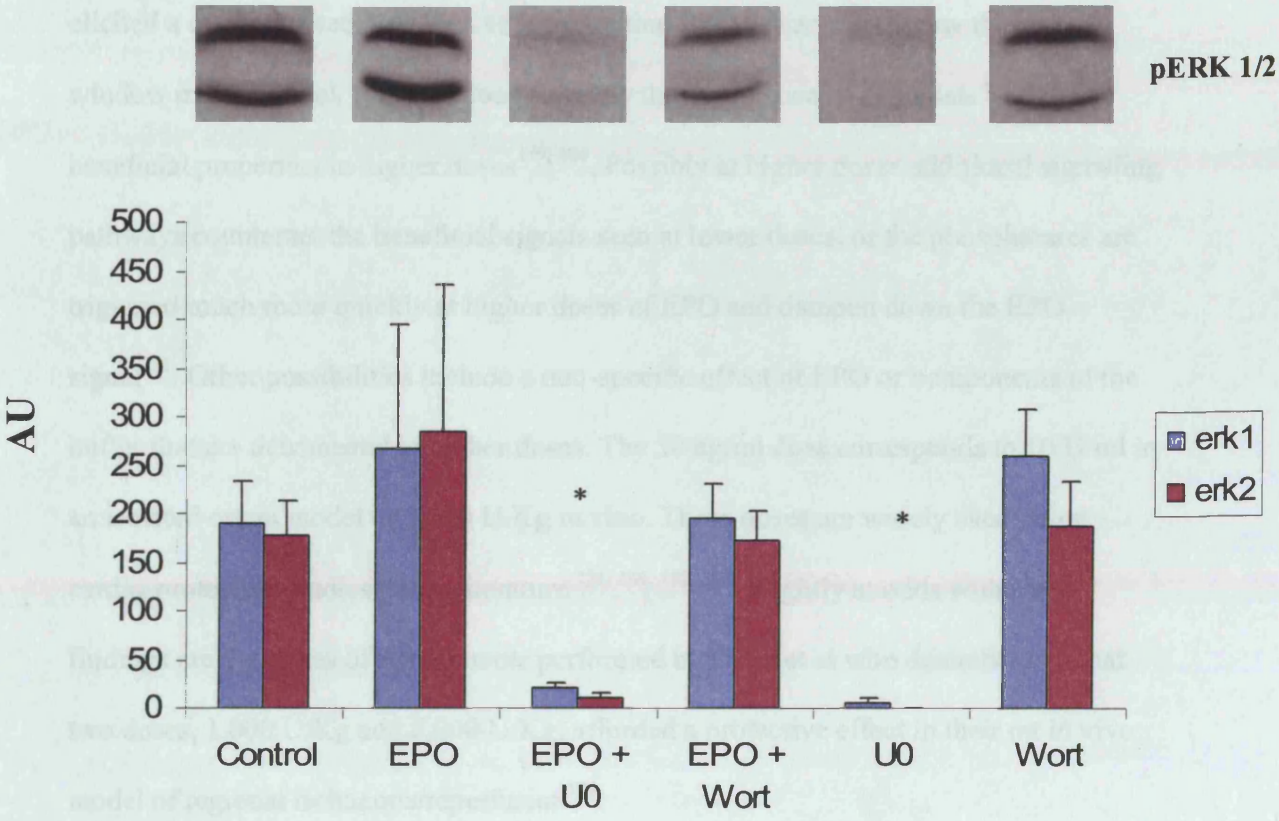


Figure 4. 9, The effect of a 50ng/ml dose of EPO on phospho-ERK levels in a model of global ischaemia. Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ v Control

4.4 Discussion

4.4.1 Dose Response

The data outlined within the results confirms that EPO can limit the cardiac infarct size in a model of reperfusion-injury when administered after a period of ischaemia in an isolated perfused heart model. The dose response clearly demonstrates that a 50 ng/ml

dose is the most effective. Indeed, only the 50 ng/ml dose out of all those measured elicited a cardio-protective effect suggesting that EPO has a very narrow therapeutic window in this model. It is a curious anomaly that some receptor agonists lose their beneficial properties in higher doses[179, 180]. Possibly at higher doses additional signalling pathways counteract the beneficial signals seen at lower doses, or the phosphatases are triggered much more quickly at higher doses of EPO and dampen down the EPO signal[181]. Other possibilities include a non-specific effect of EPO or components of the buffer that are detrimental at higher doses. The 50 ng/ml dose corresponds to 10 U/ml in an isolated organ model or 5,000 U/Kg in vivo. These doses are widely used in rat cardio-protective studies in the literature [121, 146, 182, 183]. Slightly at odds with these findings are the series of experiments performed by Parsa et al who demonstrated that two doses, 1,000 U/Kg and 5,000 U/Kg, afforded a protective effect in their rat in vivo model of regional ischaemia/reperfusion[184].

4.4.2 Involvement of RISK Pathway

Confirmation that the cardioprotection is caused by EPO, rather than the vehicle in which the protein is dissolved, is provided by the data indicating abolishment of protection when EPO is denatured by boiling for 10 minutes. The EPO vehicle contains urea, glycine, polysorbate 20, calcium chloride, a complex of 5 amino acids and phosphate buffer. However, the suppliers of the drug were not willing to specify the exact component of the drug.

The cardioprotective effect of EPO in these experiments seems to be as a result of a direct action on the heart. Indeed, the isolated perfused heart model uses a crystalloid buffer rather than blood, and is mediated by the known protective signalling moieties PI3K and ERK 1/2, as shown by the ability of their respective inhibitors to abolish EPO-mediated protection. Activation of these kinases has been shown to provide protection at reperfusion by many other pharmacological agents, as described in chapter 1. Thus the results were as hypothesised. What was not predicted was that L-NAME would fail to block the protection when co-administered with EPO. It should be noted, however, that the control L-NAME group had similar infarct sizes. NO production has been considered to be beneficial at reperfusion, albeit less effective than NO donation prior to ischaemia[185]. However, Shi et al discovered that L-NAME failed to block EPO-mediated cardioprotection, albeit when administered prior to index ischaemia[153]. It was even found by Woolfson et al that a continuous perfusion of L-NAME throughout the perfusion protocol actually reduced infarct size[186]. In our model L-NAME cannot be providing protection in its own right in this setting or the activation of eNOS by EPO would be detrimental, which is clearly not the case here. One possible explanation is that nitric oxide produced from a different source to eNOS is combining with the ROS present at reperfusion to form the pro-injury peroxynitrite. The haemodynamic data provided no distinctive features for the L-NAME groups nor did the risk volumes or animal sizes. Therefore, nitric oxide must be playing no contributory part (as suggested by Shi), nitric oxide is detrimental at reperfusion in this model or the infarct data provided is misleading and requires an additional study to directly compare the co-administration of EPO and L-NAME with EPO administered solely, rather than incorporated as part of a larger study.

Western blot analysis showed non-significant increases in phosphorylation of both ERK and Akt in the EPO group compared to control. However, EPO produced a significant 2-fold increase in phosphorylation of eNOS, which is known to be downstream of Akt in the signalling cascade [187, 188]. The inability of L-NAME to significantly block EPO-mediated protection at reperfusion suggests that, despite being phosphorylated, eNOS has no functional role in the protective pathway in this model. Within the experience of our group, the use of pharmacological intervention at reperfusion, but not prior to ischaemia, does not always provide unequivocal blots establishing significant increases in kinase phosphorylation of Akt and ERK despite a clear ability for the inhibitors of these kinases to eliminate the drug-induced protection. In an attempt to try and prevent non-viable cells skewing the results, a global ischaemia model incorporating a shorter ischaemic period was used. In addition a shorter period of reperfusion was included in this global ischaemia protocol in order to ensure that the window of phosphorylation might not be missed. However, the results from this global ischaemia protocol proved to be very similar to that of the regional ischaemia.

There are a number of possibilities that may account for the difficulty in observing increased phosphorylation of kinases by Western blot that the pharmacological inhibition studies suggest should be involved:

1. The drugs that have a tendency to provide inconclusive results in a laboratory setting are agonists for extracellular receptors such as GLP-1 and leptin. The

signals passed on from these receptors may be insufficient to be demonstrated on a technique as insensitive as Western blotting.

2. There could be a small window of activation around the commencement of reperfusion. By the time the samples were taken, i.e. after 10 and 15 minutes of reperfusion, the signal may have become dissipated or regulated by phosphatases. The use of global ischaemia and a shorter reperfusion time point for the Western blots did not resolve the issue any further.

3. In order to elicit cardioprotection, EPO may only require a small increase in phosphorylation of the target kinases compared to the control. Such an increase in phosphorylation may be too small to be detected by a technique as crude as Western blotting.

4. Standard protocols were used within the group as recommended by suppliers of the antibodies (Cell Signalling Technology and Santa Cruz), but there potentially could have been some defect within the protocol, the antibodies or operator error. However, the Western blotting procedure was repeated at least 4 times with much care for detail (new antibodies, preparation of new samples, changes in the moment of collecting the samples) with similar results.

It is worth noting that none of the 3 papers published thus far demonstrating EPO-mediated protection at the point of reperfusion have included Western blots showing an

increase in kinase phosphorylation as a result of EPO administration at reperfusion [183, 189, 190]. Those publications where EPO has been shown to successfully and significantly increase kinase phosphorylation have almost exclusively been as a result of EPO administration prior to index ischaemia. This fact may be coincidental or it may be as a result of a general difficulty in establishing the activation pattern of these pro-survival kinases at reperfusion.

4.4.3 EPO at Reperfusion In Vivo

In tandem with these experiments, Paul Govewalla from our group performed similar experiments administering a 5000 U/Kg dose of EPO at reperfusion in a rat open chest model of ischaemia-reperfusion. Similarly, he showed that EPO significantly limited infarct size, an effect that was abolished totally by wortmannin and partially by LY294002 [191]. Importantly, these in vivo studies included a reperfusion period of 24 hours, indicating that the beneficial effect of EPO is not merely an artefactual delaying of cell death, but a long-term salvage of the non-infarcted tissue.

4.5 Conclusion

Reperfusion is necessary to salvage ischaemic myocardium from cell death. However reperfusion has been shown to exacerbate the injury caused by ischaemia[192]. Therefore, reliable intervention to limit reperfusion-induced cell death will improve patient long-term prognosis following myocardial infarction. The RISK pathway has recently been proposed as a method of protection against reperfusion injury[61] that includes two key mediators, PI3K/AKT and ERK 1/2. EPO has been shown to activate these signalling moieties[126] making EPO a viable candidate agent to limit reperfusion injury.

As an established clinical treatment, EPO may represent a viable therapeutic option in the reperfusion setting as an adjunct to a thrombolytic agent or administered solely during angioplasty.

Chapter 5 - EPO within a Cellular Model

5.1 Aims and Protocols

Cellular models have long been used as an alternative method of examining similar phenomena in order to gain a deeper understanding of the cellular mechanics involved. In this instance, cell death as a consequence of hypoxia/re-oxygenation and protection from this death by pharmacological means were the aims of the following studies.

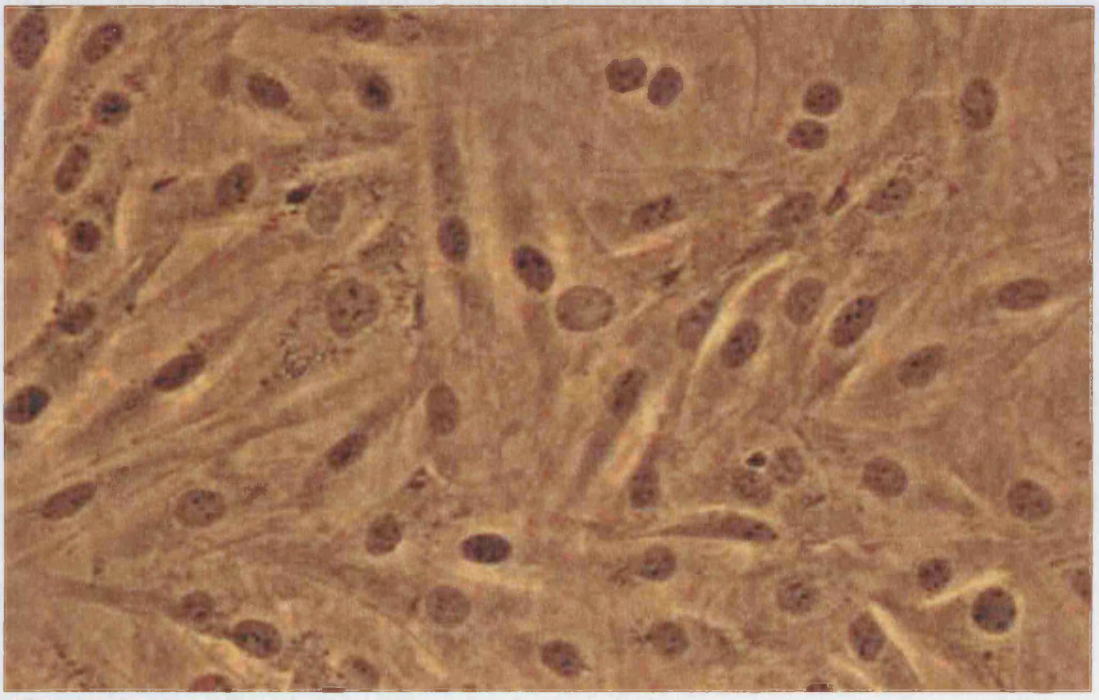


Figure 5. 1, H9c2 cells in culture under 20x magnification light microscope

We decided to use the H9c2 cell line as a cellular model for hypoxia/reoxygenation studies, as it is a well-known undifferentiated neonatal rat cardiomyoblast cell line [193, 194]

that has been used previously to examine hypoxic cell death and prevention of injury by hypoxia [195]. Prior to using EPO in this cellular model, we initially thought it would be prudent to assess whether we could demonstrate protection in this model using a tried and tested cardio-protective agent, namely atorvastatin[72, 73, 196, 197].

Taking into account the complications related to the identification of the contribution of apoptosis and necrosis to the infarction in an intact heart, discussed in section 1.2, we decided to use a cell model in order to differentiate between these two forms of cell death and thereafter to use sensitive and specific inhibitors in order to examine the mechanism of death and the possibility of increasing cell survival. To achieve this, in the first instance we attempted to characterise the level of cell death in relation to the duration of hypoxia and re-oxygenation. Thereafter we choose the experimental protocol that induced sufficient cell death to allow protection to be seen but not so much as to make prevention of death impossible.

5.1.1 Experimentation

In order to replicate the ischaemia/reperfusion setting, a model of hypoxia/reoxygenation using a hypoxic chamber was established as described in chapter 2. Flow cytometry measurements were performed using a Partec flow cytometer, see chapter 2.The marker of necrosis, propidium iodide (PI), and of apoptosis, annexin v, were used to establish the quantity and type of cell death, as described in chapter 2.

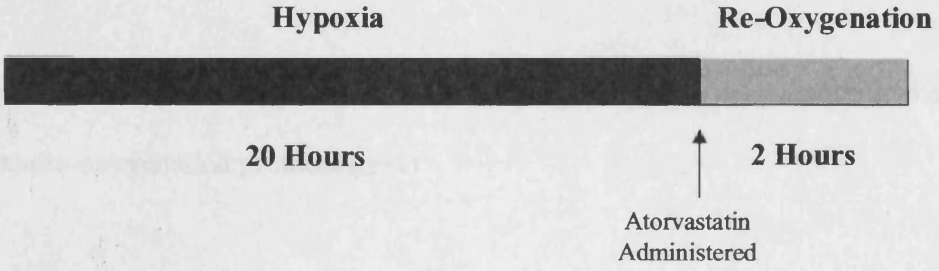
Cells underwent the following protocol:

Normoxic Protocol

Normoxia

22 Hours

Hypoxia/Re-oxygenation Protocol



The following groups were used:

1. Normoxic control – Cells kept in normoxic culture medium and conditions for 22 hours (n=3)

2. Hypoxia/Reoxygenation – Cells underwent 20 hours hypoxia and 2 hours reoxygenation (n=4)

3. Atorvastatin – Atorvastatin (50 μM) was administered at re-oxygenation in the hypoxia/re-oxygenation protocol (n=5)

5.2 Results

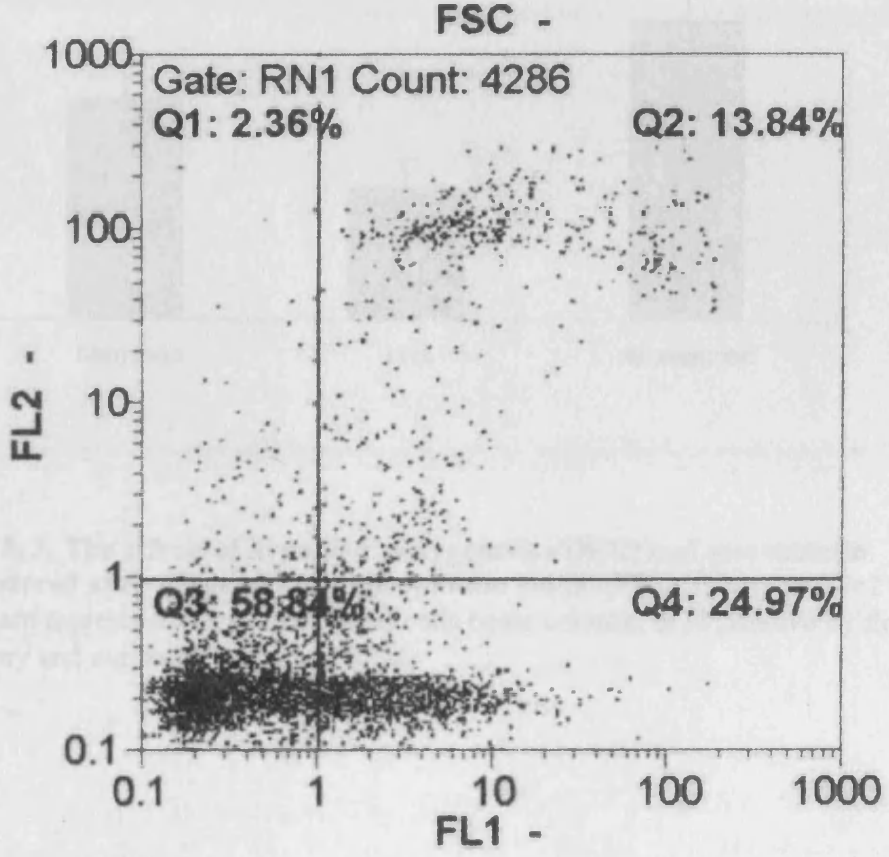


Figure 5. 2, The dot plot raw data produced by the flow cytometer. FL1 detects green fluourescence and therefore annexin v positive cells. FL2 detects red fluourescence and therefore propidium iodide positive cells. Cells that are 'apoptotic' appear in the Q4 segment as they stain for annexin only. Cells that are 'necrotic' appear in the Q2 segment as they are positive for both annexin and propidium iodide (annexin enters the holes in the necrotic cell membrane and stain phosphatidyl serine on the inner leaflet of the membrane). Cells that are negative for both annexin and propidium iodide appear in Q3 and are considered unstained or viable.

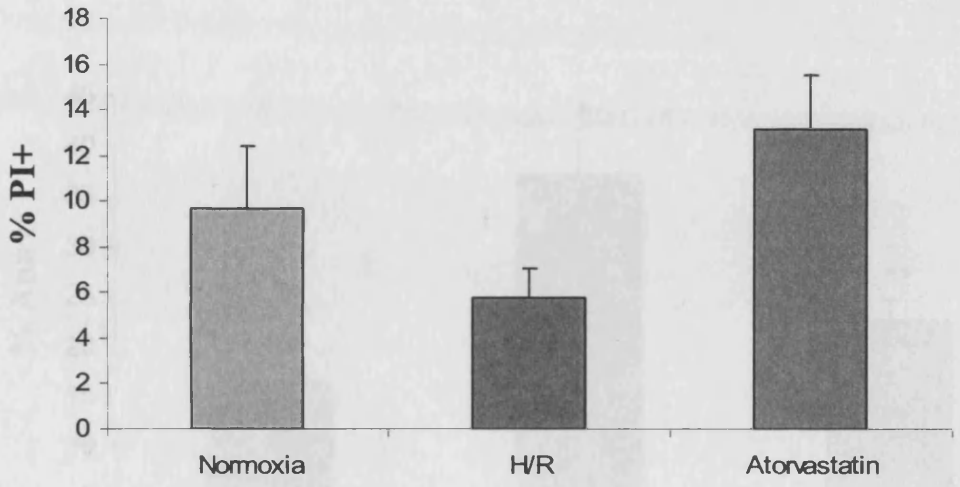


Figure 5. 3, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on propidium iodide (PI) positivity in H9c2 cells. Values are represented as percentage of cells being counted as PI positive by flow cytometry and expressed as mean ±SEM.

5.2.1 Propidium Iodide

As demonstrated in figure 5.3, there was no significant difference in PI positivity between the normoxic and hypoxia/re-oxygenation (h/r) groups (%PI+ 5.81 ±1.2 v 9.72 ±2.7). There was no significant difference between the atorvastatin and h/r groups (%PI+ 13.18% ±2.4 v 9.72 ±2.7).

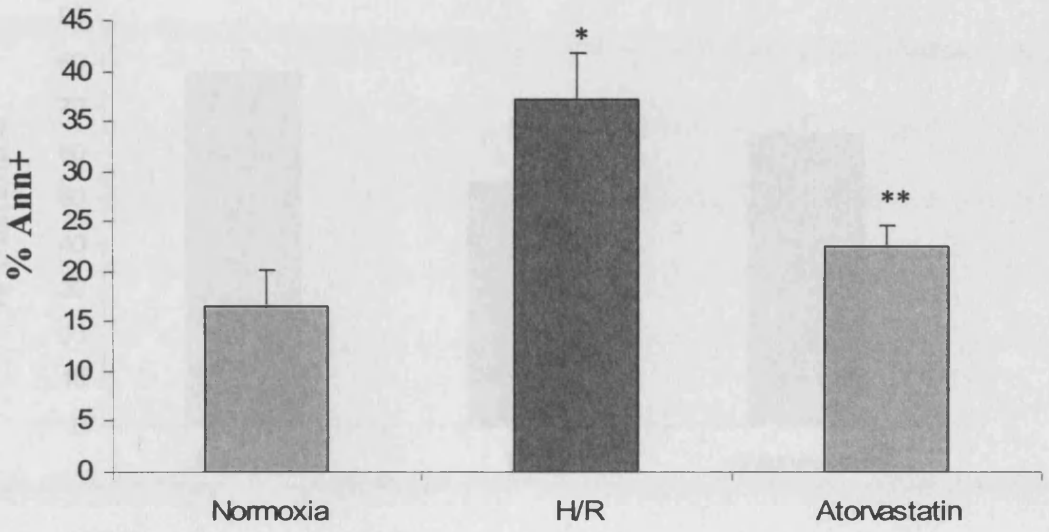


Figure 5. 4, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on annexin v (ann) positivity in H9c2 cells. Values are represented as percentage of cells being counted as PI positive by flow cytometry and expressed as mean ±SEM, * $p<0.05$ vs normoxia ** $p<0.05$ H/R

5.2.2 Annexin v

There was a significant difference in annexin v positivity between the normoxic and h/r groups (% ann+ 16.55 ±3.6 v 37.05 ±4.7, $p<0.05$). There was a significant difference between the atorvastatin and h/r groups (% ann+ 22.56 ±2.07 v 37.05 ±4.7, $p<0.05$). There was no significant difference between the normoxic and atorvastatin group.

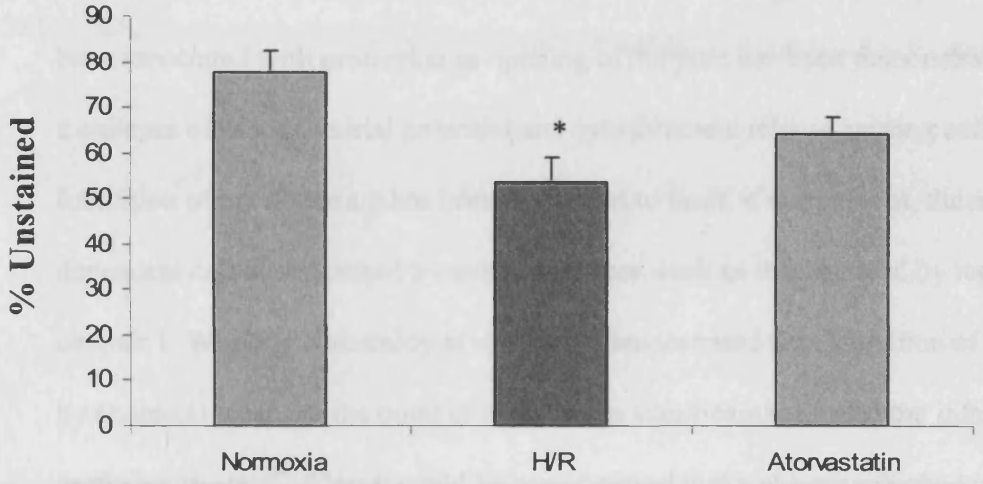


Figure 5. 5, The effects of hypoxia/re-oxygenation (H/R) and atorvastatin administered at re-oxygenation on cell viability. Values are represented as percentage of cells being counted as unstained by both PI and annexin v by flow cytometry and expressed as mean ±SEM, * $p<0.05$ vs normoxia

5.2.3 Viable Cells

Cells that stained for neither annexin v nor PI were considered to be viable. There was a significant difference in the percentage of unstained cells in the sample between the normoxic and h/r groups (% 77.64 ±4.7 v 53.38 ±5.6, $p<0.05$). There was no statistical difference between the atorvastatin and h/r groups (% 64.27 ±3.7 v 53.38 ±5.6).

5.2.4 The Role of mPTP Opening in EPO-mediated Protection

As discussed in section 1.4, the mitochondrial membrane permeability transition pore has been associated with protection as opening of the pore has been demonstrated to result in a collapse of mitochondrial potential and cytochrome c release causing cell death[50]. Inhibition of pore opening has been suggested to limit, if not prevent, the mitochondrial-dependent cell death caused by oxidative stress, such as that induced by reperfusion, see chapter 1. Work by Hausenloy et al clearly demonstrated that inhibition of pore opening by chemical means at the point of reperfusion significantly limited the infarct size in rate perfusion model[198]. Thus it could be hypothesised that a pharmacological intervention that had the effect of preventing or delaying pore opening, directly or indirectly, may limit the injury caused by reperfusion-induced oxidative stress and death.

Subsequent to the recent independent research of Yellon's group and the Halestrap group[52, 55, 198-200], there have been a number of studies that have demonstrated a delay in mPTP opening as a result of oxidative stress by a number of compounds. One particular study by Juhaszova et al examined a series of compounds and their abilities to delay pore opening by the same method described in this chapter[201]. As this study included EPO, it was natural to assume that the data could be replicated in our hands. In addition, as EPO takes its effect by acting as an agonist to its receptor, it can also be hypothesised that any delay in pore opening seen as a result of experimentation could be as a result of the protein kinase pathways highlighted in chapter 1.

To confirm the involvement of the mPTP in mediating the protective effects of EPO, a method was used that involved utilising the lipophilic cation TMRM and its prevalence to accumulate into the mitochondria due to the membrane potential of the mitochondria [202]. In a method established by Duchen's group [203, 204], TMRM loaded in mitochondria is illuminated by a laser to provide a model of oxidative stress. The mitochondria will appear stained red due to the TMRM as long as the mitochondrial membrane remains polarised. Thus depolarisation of mitochondria can be seen as a drop out of signal. Initially, there are brief flashes of intensity which Duchen et al have demonstrated to be dependent upon local sarcoplasmic reticular calcium release [205]. There are also patchy dropouts in signal due to localised depolarisation of mitochondria. Depolarisation itself manifests as a wave usually commencing at one end of the myocytes, although it occasionally is initiated in the middle or at both ends of the myocyte simultaneously. This wave then spreads through the rest of the myocyte. Subsequent to the depolarisation there is a rapid depletion of ATP that is consumed by ATPase as a result of pore opening [203, 206]. When the ATP levels become sufficiently depleted the myocyte begins to shorten into rigor contracture.

5.3 Experimentation for mPTP Opening

Myocytes were isolated from four different Sprague Dawley rats and prepared for use as described in section 2.6.

Myocytes were incubated with TMRM (3μM) for 15 minutes and 10 minutes with the drug(s) prior to undergoing the oxidative stress protocol. The following drug protocols were used:

1. Control – where no drug was administered (n=21)

2. EPO – administered at a dose of 50 ng/ml (n=29) and in combination with 0.01% DMSO vehicle control (n=7)

3. L-NAME – the NOS inhibitor, was administered at a dose of 100 μM both solely (n=11) and in combination with 50 ng/ml EPO (n=22)

4. Wortmannin – the PI3K inhibitor, was administered at a dose of 200 nM both solely (n=16) and in combination with 50ng/ml EPO (n=14)

5. U0126 – the ERK 1/2 inhibitor, was administered at a dose of 10 μM both solely (n=14) and in combination with (n=8)

Suitable healthy appearing cells were identified by light microscope and scanned by laser until all cells within the field had commenced rigor contracture.

The time to the initiation of the wave of depolarisation and rigor were measured independently. As the time to depolarisation has been shown to be affected by such environmental conditions as laser light strength and oxygen concentration, all results for each isolation were normalised against the control of that isolation such that the average for the control was 1 in order to eliminate inter-experimental variations.

5.4 Results

5.4.1 mPTP Opening

As can be seen from figure 5.6 the progression from normal healthy cell through local depolarisation and general wave of depolarisation to final rigor is quite marked. The time points measured include when the wave of depolarisation becomes first identifiable and when cell shortening first commences.

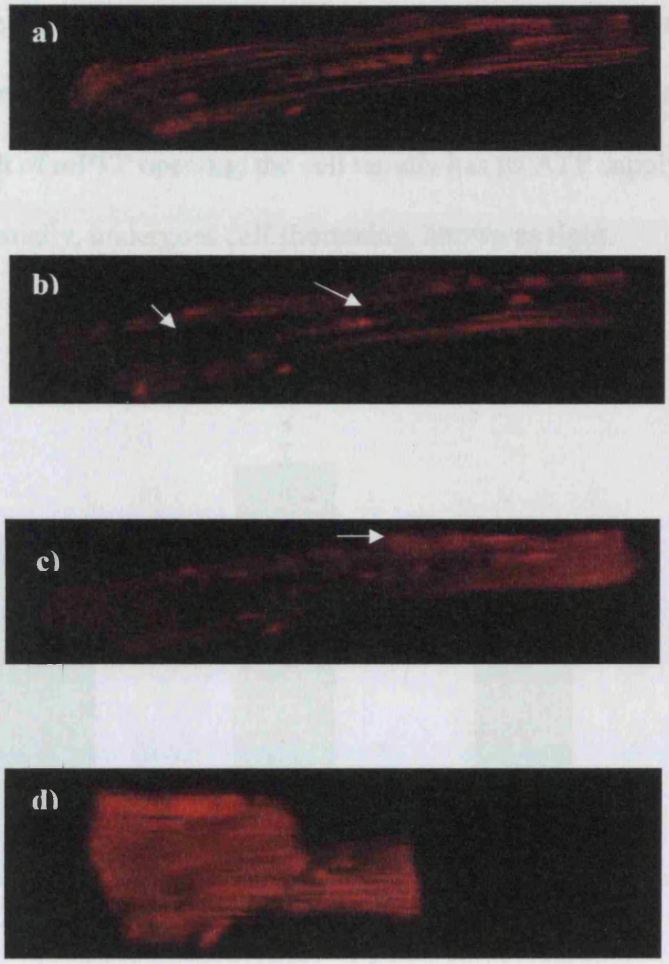


Figure 5. 6, Representation of the myocytes throughout confocal laser experimentation

Confocal images of myocytes loaded with TMRM and exposed to an argon laser.

a) Represents the myocytes at the commencement of the experiment with the TMRM-loaded mitochondria clearly lined up in striata along the length of the cell.

b) Demonstrates the drop-outs that occur in mitochondria signal after a period of exposure to oxidative stress caused by individual mitochondria depolarising completely (see arrows).

c) Subsequent to dropping out of signal in the individual mitochondria a general wave of depolarisation occurs, usually commencing from one end of the cell (see arrows).

d) As a result of mPTP opening, the cell rapidly has its ATP supply depleted and, consequentially, undergoes cell shortening, known as rigor.

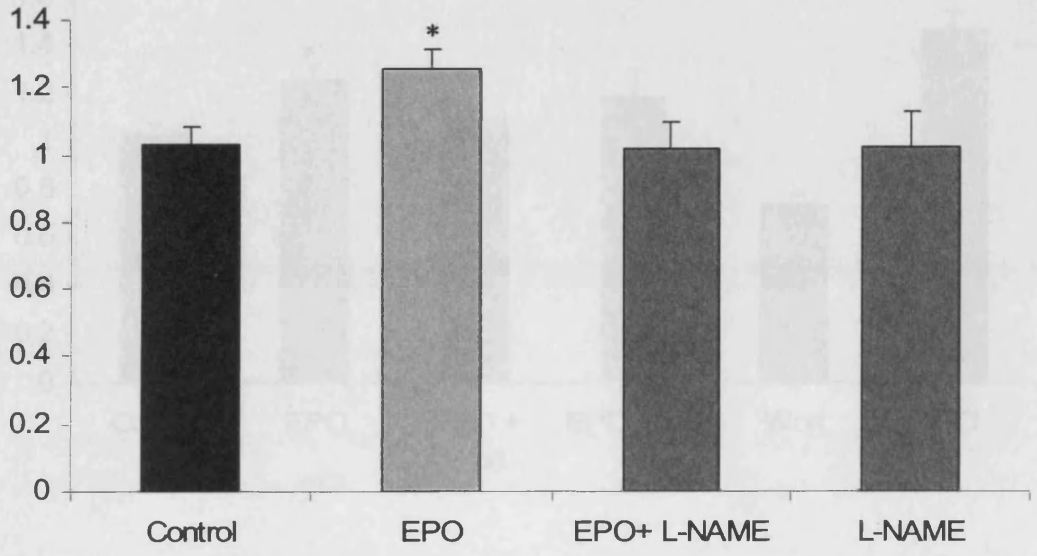


Figure 5. 7, The effect of EPO (50 ng/ml) and inhibition of NOS by L-NAME (100 μM) on the time to depolarisation due to oxidant stress. Values are represented as arbitrary units normalized against control (represented as 1) and expressed as mean ±SEM. * $p<0.05$ vs control, EPO+L-NAME and L-NAME

5.4.2 Effect of EPO and L-NAME on mPTP Opening

As can be seen from figure 5.7, EPO significantly increased the time to depolarisation versus control (1.000 ±0.051 v 1.255 ±0.059, $p<0.05$), thus the time to depolarisation was increased by 25.5%. The addition of L-NAME to EPO significantly reduced the time to

depolarisation back to control levels (1.016 ±0.83 L-NAME + EPO v 1.255 ±0.059 EPO, $p<0.05$). L-NAME administered solely seemed to have no significant effect compared with control (1.026 ±0.102 v 1 ±0.051).

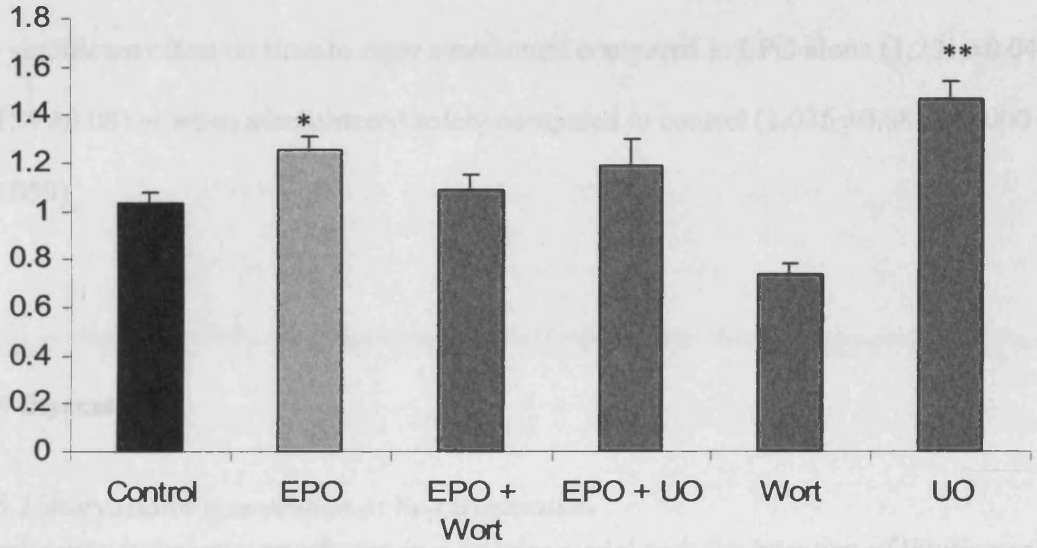


Figure 5. 8, The effect of EPO (50 ng/ml) and inhibitors of PI3K by wortmannin (Wort,100 nM) and ERK 1/2 by U0126 (UO, 10 μM) on the time to depolarisation due to oxidant stress. Values are represented as arbitrary units normalized against control (represented as 1) and expressed as mean ±SEM. * $p<0.05$ vs control, EPO+Wort and Wort. ** $p<0.05$ vs all other groups.

5.4.3 The Involvement of PI3K and ERK 1/2 in EPO-mediated Delay of mPTP Opening

The effect of administering the PI3K blocker wortmannin in combination with EPO is to significantly reduce the time to depolarisation (1.086±.069 v 1.255 ±.059, $p<0.05$). Wortmannin administered on its own seemed to shorten the time to depolarisation versus

control (0.742 ±.047 v 1.000 ±.051, $p<0.05$). The ERK 1/2 inhibitor, U0126, was given in combination and seemed to have no significant effect on EPO- mediated delay of mPTP opening (1.190 ±0.11 v 1.255 ±.059). However, U0126 when administered on its own seemed to have a distinct delaying effect on depolarisation compared to control (1.469 ± 07 v 1.000 ±0.051, $p<0.05$). The presence of wortmannin in combination with EPO had no significant effect on time to rigor contracture compared to EPO alone (1.221 ±0.046 v 1.154 ±0.08) or when administered solely compared to control (1.076 ±0.081 v 1.000 ±0.059).

5.5 Discussion

5.5.1 Atorvastatin Intervention at Re-Oxygenation

Replicating ischaemia/reperfusion in a cellular model with the intention of limiting cell death as a result of that ischaemia/reperfusion has been successfully achieved in a number of studies. In the H9c2 ventricular derived cell line, markers of cell death such as MTT staining[195], lactate dehydrogenase release[207], propidium iodide accumulation[208], morphology[209], caspase 3 and PARP cleavage[210] have been used. These studies have exclusively examined intervention that was activated prior to index hypoxia. In our model we have attempted to use the cellular model to replicate intervention at re-oxygenation using atorvastatin, a drug that has been shown to protect the myocardium directly, both when administered prior to ischaemia[211] and at the point of reperfusion [72, 73]. As can be seen from the data, the propidium iodide results showed no significant difference between any of the groups. As mentioned in the methods chapter, propidium

iodide (PI) is meant to be a marker for necrosis. In theory PI should measure cells damaged during hypoxia as necrosis is an ATP and oxygen independent method of cell death. It would therefore be expected that the hypoxia/re-oxygenation group would have a higher level of PI positive cells compared to control even if atorvastatin failed to limit such increase in PI positive cells. It must be concluded from this data, therefore, that the hypoxia/re-oxygenation protocol was providing insufficient stimulus for cell death. However, the annexin v results demonstrated that hypoxia/reoxygenation produced an increase in the number of annexin positive cells with atorvastatin limiting this increase. This result would be entirely expected especially as annexin v positivity is associated with apoptosis and reperfusion/re-oxygenation injury.

So why did we get strong predictable results for annexin v but not PI? A study by Eijnde indicated that phosphatidyl serine becomes expressed on the surface of H9c2 cells during myotube formation[212]. Since phosphatidyl serine is the cell surface marker that annexin binds to, any data that includes annexin v staining in this H9c2 cell line cannot be relied upon as hypoxia/re-oxygenation could alter the cell surface expression of phosphatidyl serine independently of apoptosis. Many other periods of both hypoxia and reoxygenation were used. However, it proved impossible to find a period of hypoxia and re-oxygenation whereby either sufficient cell death was caused by re-oxygenation alone to allow an agent to limit that injury successfully, or cell death was so extreme as to preclude the possibility to limit any injury at all. In addition many other markers of cell death were used, including MTT, Trypan Blue, TUNEL and the fluorescent marker of caspase 3 cleavage, phiphilux. The experiments were repeated using cells that had been caused to

differentiate by limiting the foetal calf serum in the medium, in addition to using a primary culture of rat ventricular myocytes, although the myocytes proved too fragile to use in this model. None of these experiments provided a robust reproducible model. It was for this reason that we decided that an alternative cellular model of reperfusion injury should be used, and as such we undertook this in a series of confocal microscopy studies.

5.5.2 EPO-mediated Delay in mPTP Opening

Inhibiting the opening of the mPTP has been demonstrated to limit infarct size both prior to index ischaemia and at reperfusion in a Langendorff model of ischaemia/reperfusion[51]. It can be deduced, therefore, that by preventing or delaying pore opening you can limit reperfusion-induced injury. This confocal microscope model mimics reperfusion by causing oxidative stress as previously mentioned ,see methods chapter, section 2.6.1, as such a delay in pore opening would be interpreted as a beneficial effect against reperfusion injury and indicate that the protective effect by EPO at reperfusion would be achieved via the mPTP. Our results demonstrate that EPO clearly delays opening of the mPTP. The PI3K inhibitor wortmannin abolishes the protective effect of EPO, suggesting that PI3K is involved in this protection that leads to an inhibition of the mPTP opening. These findings are in line with research by Juhaszova et al who not only showed that EPO could delay pore opening but also found that wortmannin could abolish a beneficial delaying effect using insulin as the protective agent[201]. The exact mechanism by which PI3K, and its downstream kinase Akt, limit pore opening is currently poorly understood. The suggestion put forward by Juhaszova et al is that Akt functions by inhibition of glycogen synthase kinase-3β (GSK 3β). However, they could not elucidate

precisely how inhibition of GSK 3β would lead to an inhibition of pore opening, although they did hypothesise that GSK 3β phosphorylates and inactivates Bcl-2. There is some suggestion that Bcl-2 could raise the ROS threshold to which the mPTP is sensitive, thus inhibiting or delaying its opening[201]. However, there is no indication whether the ROS levels in the TMRM confocal study are physiological and therefore directly relevant when it comes to ROS threshold levels. Similarly, the Bcl family members Bax and BAD have been suggested as being mPTP openers[213]. Both these molecules are known targets of Akt[214], which inactivates them through phosphorylation.

One other possible mechanisms by which PI3K/Akt could possibly lead to inhibition of pore opening is by nitric oxide release via the downstream target of PI3K/Akt, eNOS.

5.5.3 The Effect of U0126

The U0126 data is surprising and unexpected. As U0126 is an inhibitor of ERK 1/2, a kinase which when strongly activated is known to be beneficial at reperfusion, it was expected that the use of U0126 would abolish the protective effects of EPO. However, what the data demonstrates is that the concomitant administration of EPO and U0126 results in a similar time to depolarisation as EPO by itself. It might be concluded from this data that ERK 1/2 was not involved in the inhibition of mPTP opening if the sole administration of U0126 had given a similar time to depolarisation as the control. However, surprisingly, we saw that U0126 by itself increases the time to depolarisation much more than EPO alone.

There are a myriad of possibilities to explain the phenomenon:

1. *Operator error* is the most obvious solution, but it can be seen from figure 5.9 that an independent observer found exactly the same phenomenon in a completely different set of experiments, suggesting that operator error is an unlikely explanation.

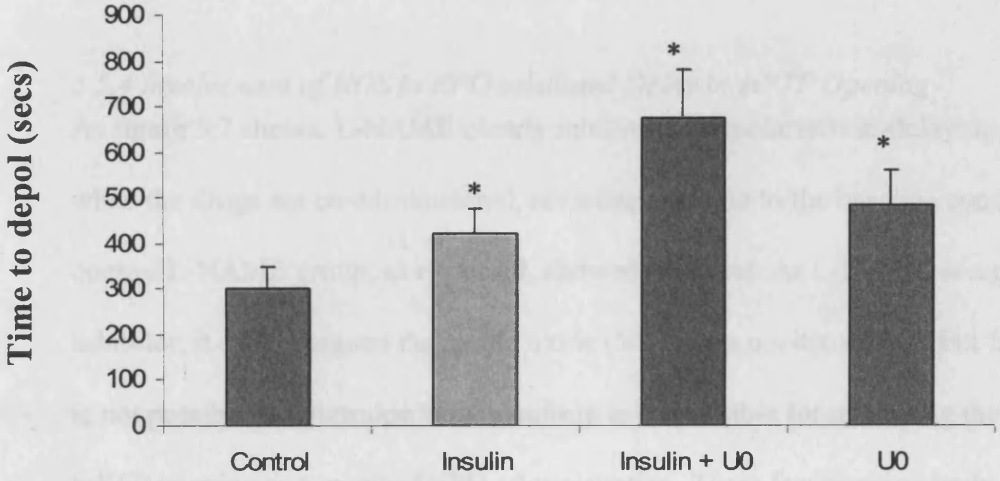


Figure 5. 9, Confocal microscopy experiment using exactly the same protocol as mentioned in section 5.3, performed by an independent observer. Values are represented time to depolarisation wave initiation measured in seconds expressed as mean ±SEM. * $p<0.05$ vs control

2. *UO126 solubility*. U0126 is a substance that does not dissolve in aqueous solutions very easily. Even when pre-dissolved in DMSO the drug can still precipitate when being dissolved in an aqueous solution. It is therefore possible that some precipitate of the drug could be diffracting the laser beam and thus reducing the oxidant stress which the myocytes undergoes, resulting in a delayed time to the wave of depolarisation.

3. *Increase in Ca2+ levels* are known to decrease TMRM fluorescence so it is possible that U0126 caused an increase in Ca2+ and, by reducing the intensity of TMRM, also reduced the oxidant stress.

4. ERK inhibition within this model could be protective where it is not used in vivo or in an isolated perfusion model, although exactly how this might be manifest seems unclear, making the possibility unlikely.

5.5.4 Involvement of NOS in EPO-mediated Delay in mPTP Opening

As figure 5.7 shows, L-NAME clearly inhibits the depolarisation-delaying effects of EPO when the drugs are co-administered, reverting the time to the baseline control level. The control L-NAME group, as expected, showed no effect. As L-NAME is a general NOS inhibitor, it can be argued that nitric oxide (NO) has a positive effect, but from this data it is not possible to determine which isoform is responsible for mediating the delay in mPTP opening as a result of EPO administration. There is a growing body of data studying the effect of NO on the mPTP. One pivotal study by Brookes et al used the NONOate group of nitric oxide donors to examine the effect NO on cytochrome c release, membrane polarisation and calcium efflux from rat liver mitochondria [215]. They found that NO inhibited mPTP opening with an IC_{50} of 11 nM, which represents a physiological concentration. In addition they saw reduced cytochrome c release, reduced mitochondrial Ca^{2+} accumulation and mitochondrial membrane depolarisation. This effect has been largely replicated in bovine endothelial cells by Dedkova and Blatter[216] and in mouse striatal neurons by Horn et al[217], thus indicating that the effect is not tissue specific. This common appearance of depolarisation may well be a key occurrence. Ichas et al described a common intracellular calcium spike that appeared when mitochondrial depolarisation occurred [218]. The source of the calcium has been identified as mitochondrial [219] and attributed to a 'transitional' opening of the mPTP[219, 220]. It has been

suggested that this form of reversible mPTP opening works as an energetically favourable Ca^{2+} release valve, preventing a dangerous over-accumulation of Ca^{2+} within the mitochondrion[221]. Dedkova and Blatter argue that the depolarisation itself actually limits influx and efflux of calcium and that in this way over-accumulation of calcium within the mitochondria does not occur. It is important to note that during transitional mPTP opening mitochondrial swelling did not occur as the pore seems impermeable to any molecule over 300 Da in size. Thus it should not be confused with 'pore opening', which is measured in these experiments, whereby cytochrome c is released and apoptosis initiated.

5.6 Conclusion

Prevention of mPTP pore opening has been demonstrated to have a direct consequence on limiting infarct size in organ perfusion heart models. Thus it seems likely that the beneficial effect can be transposed into the clinical setting. EPO is well documented to have a beneficial effect in the clinical setting and the data presented within this chapter suggests that this effect could well be due to prevention of pore opening. In addition to a myriad of other pathways, PI3K/Akt has been shown here to have a direct effect on the mPTP within the myocardium in addition to NOSs, as shown by the ability of L-NAME also to abolish the protective effect of EPO. It is entirely conceivable that protective pathways initiated by EPO, as discussed in chapter 1, have the mPTP as their end effector.

Chapter 6 - The effect of chronic EPO treatment on the myocardium

6.1 Introduction and Aims

Since the cloning of its gene and subsequent production of recombinant proteins, EPO has come to be used in chronic treatment mainly to correct anaemia as a result of such disorders as kidney or chronic heart failure, as described in chapter 1. Research relating to the clinical use of EPO demonstrates it has beneficial effects, usually ascribed to the correction of anaemia. This research has extended to cases of chronic heart failure, a known cause of anaemia. However, with recent research uncovering the beneficial effects of EPO directly on the myocardium, i.e. unrelated to the correction of anaemia, there seems to be a question regarding the nature of the effect which EPO has on patients when administered as a chronic treatment. Other studies examining the chronic effect of atorvastatin on the heart demonstrated that the phosphatase PTEN was negatively regulating the beneficial PI3K pathway, thus eliminating the protection seen when atorvastatin was administered acutely. We therefore set out to determine whether chronic administration of EPO can have a beneficial effect in a model of myocardial ischaemia/reperfusion and by what mechanism any observable effect could be achieved.

6.2 Experimental Protocol

Sprague Dawley rats were treated for 3 weeks according to the following protocols:

A. Saline injected sub-cutaneously three times a week

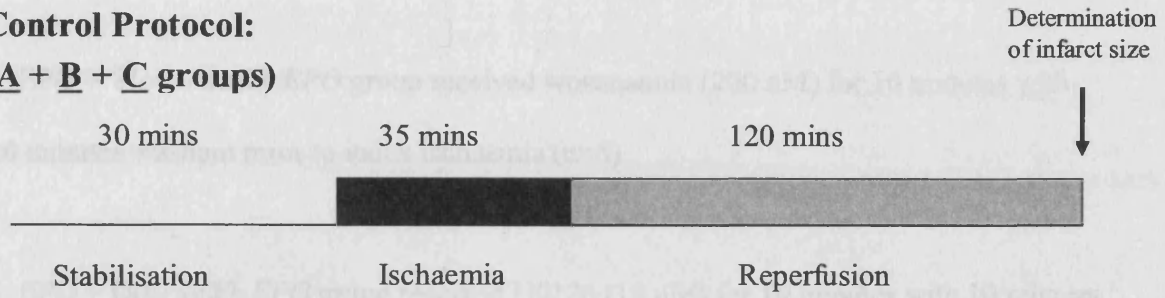
B. EPO injected sub-cutaneously at a dose of 5,000 units/kg once a week *(1xWk EPO)*

C. EPO injected sub-cutaneously at a dose of 5,000 units/kg three times a week (*3xWk EPO*)

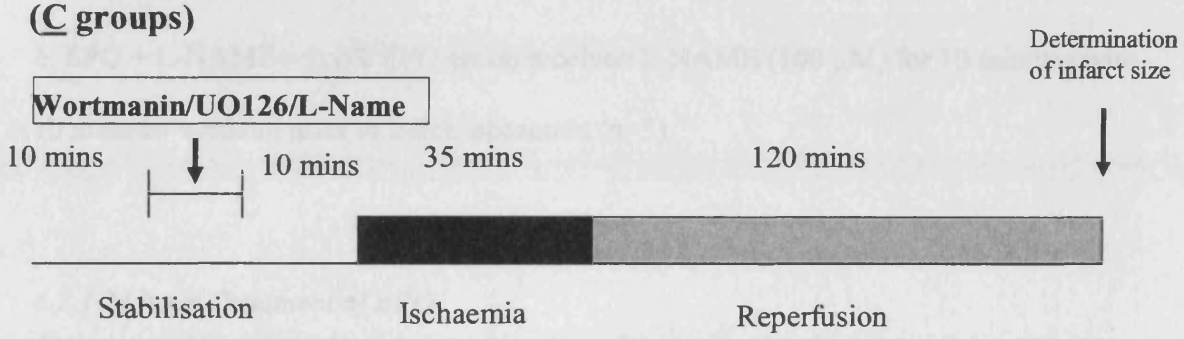
At the completion of the 3 week treatment all rats weighed between 300-400g, without any significant difference between the groups. Hearts were excised in the normal manner for perfusion on the Langendorff apparatus, and the blood was sampled and analysed for the measurements of haematocrit and plasma EPO concentrations.

Hearts were mounted on the Langendorff apparatus and put through the following perfusion protocols:

Control Protocol:
(A + B + C groups)



Inhibitor Protocol
(C groups)



In detail, the groups presented in the above figures were:

1. Saline control – saline (200 μL) was administered three times a week for three weeks (n=6)

2. *1xWk EPO* – EPO (200 μL, 5000 U/kg) was administered once a week for three weeks (n=6)

3. *3xWk EPO* - EPO (200 μL, 5000 U/kg) was administered three times a week for three weeks (n=7)

4. EPO + Wort – *3xWk EPO* group received wortmannin (200 nM) for 10 minutes with 10 minutes washout prior to index ischaemia (n=6)

5. EPO + U0 - *3xWk EPO* group received U0126 (10 μM) for 10 minutes with 10 minutes washout prior to index ischaemia (n=6)

6. EPO + L-NAME - *3xWk EPO* group received L-NAME (100 μM) for 10 minutes with 10 minutes washout prior to index ischaemia (n=5)

6.2.1 24 hour Treatment of EPO

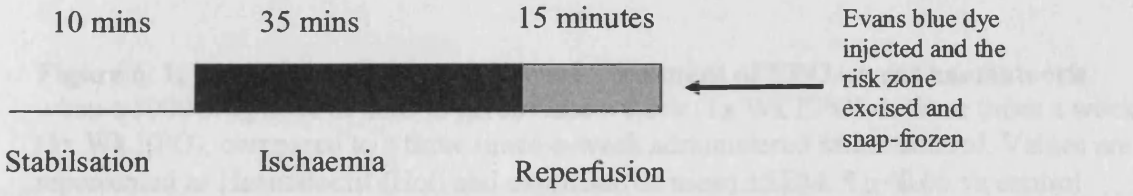
In order to determine whether any protection observed in the chronic model was due to the increase in haematocrit and, consequently, to the improved oxygen supply, we administered a single sub cutaneous dose of either 5000 Units/kg EPO (n=) or 200 μl saline (n=5) 24 hours before the index ischaemia. The Langendorff protocol was the same as per control in the chronically treated groups.

6.2.2 Western Blotting

In order to determine the phosphorylation status of certain key protein kinases, Western blots were performed as described in chapter 2. The protocols used were as follows:

Naïve – Hearts from animals which received a 3 week treatment of saline (n=5) or 3x week treatment of EPO (n=5) were excised, briefly washed in ice-cold Krebs buffer to remove blood and then snap frozen in liquid nitrogen

Standard Protocol – Hearts from animals which received a 3 week treatment of saline (n=3) or 3x week treatment of EPO (n=3) were excised and underwent the experimental protocol illustrated below:



The levels of the total and the phosphorylated Akt, ERK, eNOS, PTEN were measured. The level of total iNOS was also assessed. Results were corrected for protein loading by comparing concentrations of β-actin present in each sample.

6.3 Results

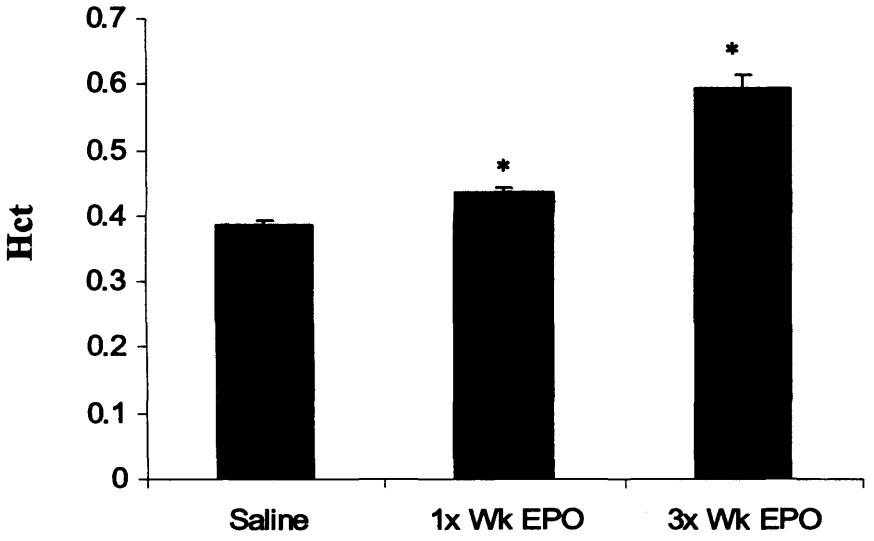


Figure 6. 1, The effect of a chronic 3 week treatment of EPO on rat haematocrit when a 5000U/kg dose of EPO is given once a week (1x Wk EPO) or three times a week (3x Wk EPO), compared to a three times-a-week administered saline control. Values are represented as Haematocrit (Hct) and expressed as mean ±SEM. * $p<0.05$ vs control

6.3.1 Haematocrit

As demonstrated in figure 6.1, the once a week dose of EPO demonstrated a significant increase in haematocrit compared to the saline control (0.436 ±.006 v 0.386 ±0.008, $p<0.05$). The 3 times a week dose had a significantly increased haematocrit compared to both the control saline group and the once-a-week dose group (0.595 ±0.018 v 0.386 ±0.08; v 0.436 ±0.06, $p<0.05$). There was no significant difference in haematocrit for the acute EPO dose group compared to the saline control (0.410 ±0.008 v 0.410 ±0.008).

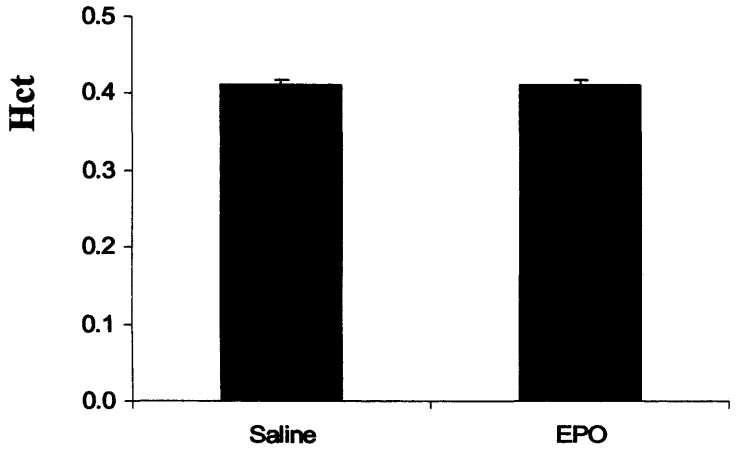


Figure 6. 2, The effect of the acute treatment with EPO on the rat haematocrit 24 hours after a single 5000U/kg dose of EPO. Values are represented as Haematocrit (Hct) and expressed as mean ±SEM.

The haematocrit content for both the saline control and acute EPO groups were identical (0.41 ±0.008), see figure 6.2.

6.3.2 Langendorff Results

Groups	Body Weight (g)	Muscle Volume (cm^3)	Risk Volume (cm^3)
Saline Control	384 ±7.5	1.107 ±0.034	0.535 ±0.025
1xWk EPO	348 ±4.9	0.996±0.032	0.526 ±0.043
3x Wk EPO	336 ±15.9	0.929 ±0.046	0.517 ±0.041
EPO + Wort	333 ±8.8	0.927 ±0.056	0.419 ±0.025
EPO + U0	382 ±2.8	0.982 ±0.054	0.545 ±0.029
EPO + L-NAME	349 ±2.3	0.913 ±0.051	0.557 ±0.037

Table 6. 1, Characteristics of animals in the chronic treatment groups

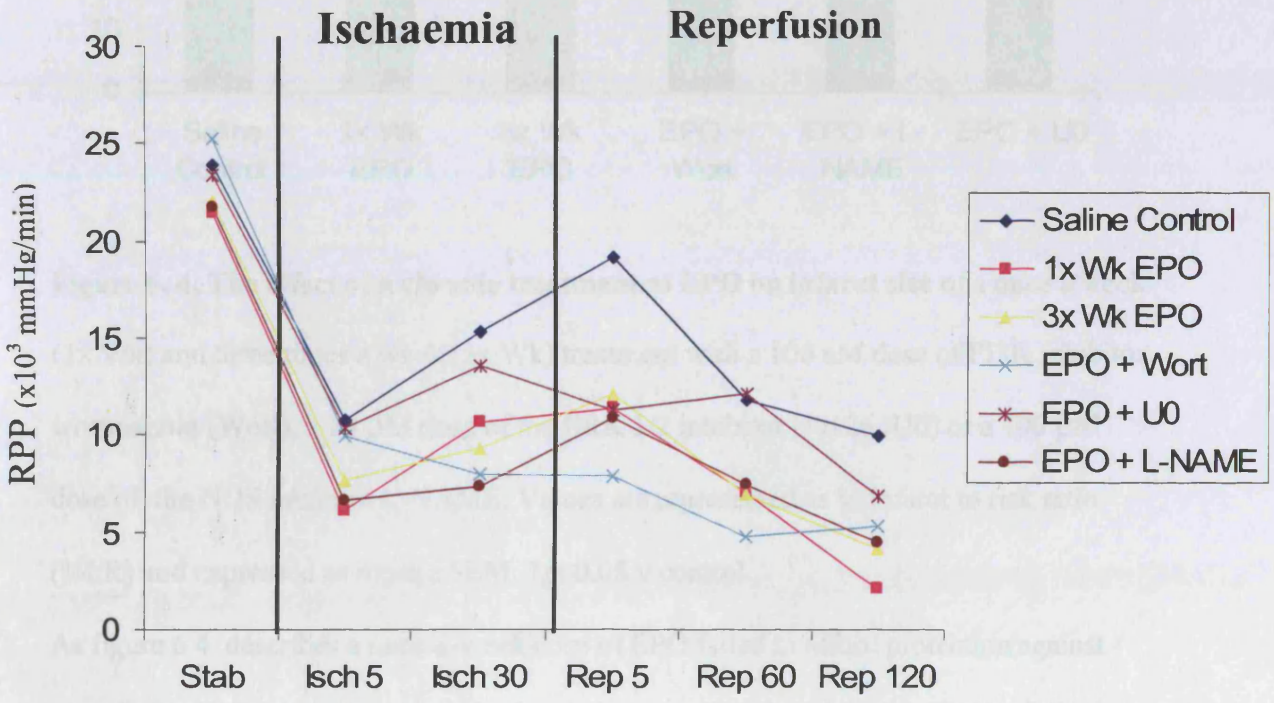


Figure 6. 3, Representation of the change in Rate-Pressure Product (RPP) throughout the experimental protocol from stabilisation (Stab) through ischaemia (Isch) and into reperfusion (Rep)

Exclusions

Two animals from the 3x Wk EPO group were excluded due to too large a risk zone, one animal from the saline control group was excluded due to too large a risk zone, one animal from the EPO+U0 group was excluded due to an infarct of less than 5%

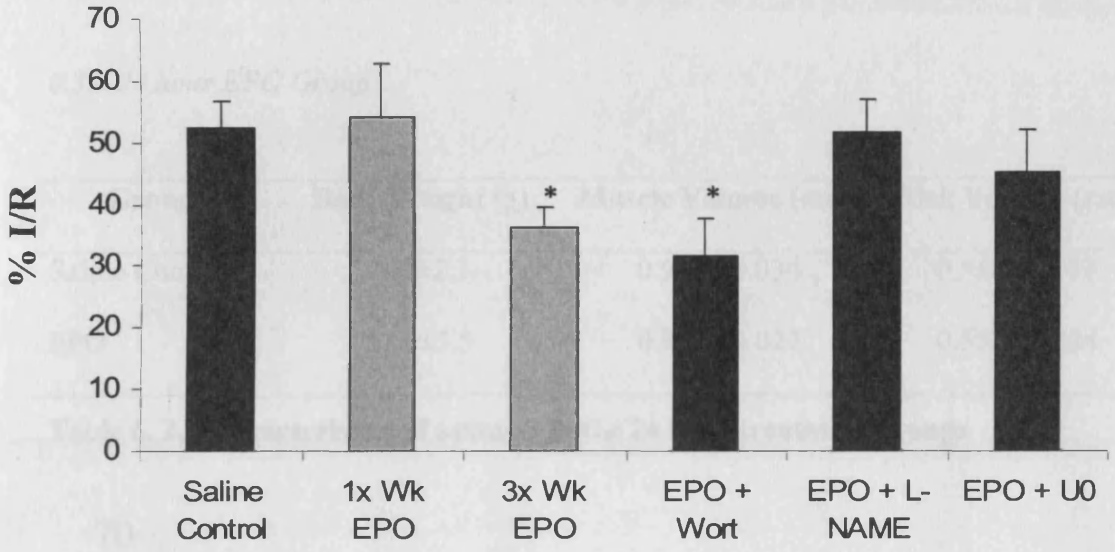


Figure 6. 4, The effect of a chronic treatment of EPO on infarct size of a once a week (1x Wk) and three times a week (3x Wk) treatment with a 100 nM dose of PI3K inhibitor wortmannin (Wort), a 10 μM dose of the ERK 1/2 inhibitor U0126 (U0) or a 100 μM dose of the NOS inhibitor L-NAME. Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *$p<0.05$ v control

As figure 6.4 describes a once-a-week dose of EPO failed to afford protection against reperfusion by comparison to a saline control group (%IR 54.07 ±8.7 v 52.25 ±4.4), whereas a three times a week regime did reduce infarct size significantly compared to control (%IR 36.23 ± 3.2 v 52.25 ±4.4, $p<0.05$). The PI3K inhibitor wortmannin did not

block the protective effect of this three times a week dose of EPO (%IR 31.7 ±6.0 v 36.2 ±3.2). However, the NOS inhibitor L-NAME did abrogate the protection provided by a three times a week regime (%IR 51.6 ±5.6 v 36.2 ±3.2, $p<0.05$). The ERK 1/2 inhibitor U0126 showed no significant difference against the EPO group (%IR 45.1 ±7.1 v 36.2 ±3.2) or the saline control (%IR 45.1 ±7.1 v 52.3 ±4.4).

6.3.3 24 hour EPO Group

Groups	Body Weight (g)	Muscle Volume (cm^3)	Risk Volume (cm^3)
Saline Control	390±2.3	0.960 ±0.034	0.510 ±0.017
EPO	373 ±5.5	0.999 ±0.022	0.552 ±0.024

Table 6. 2, Characteristics of animals in the 24 hour treatment groups

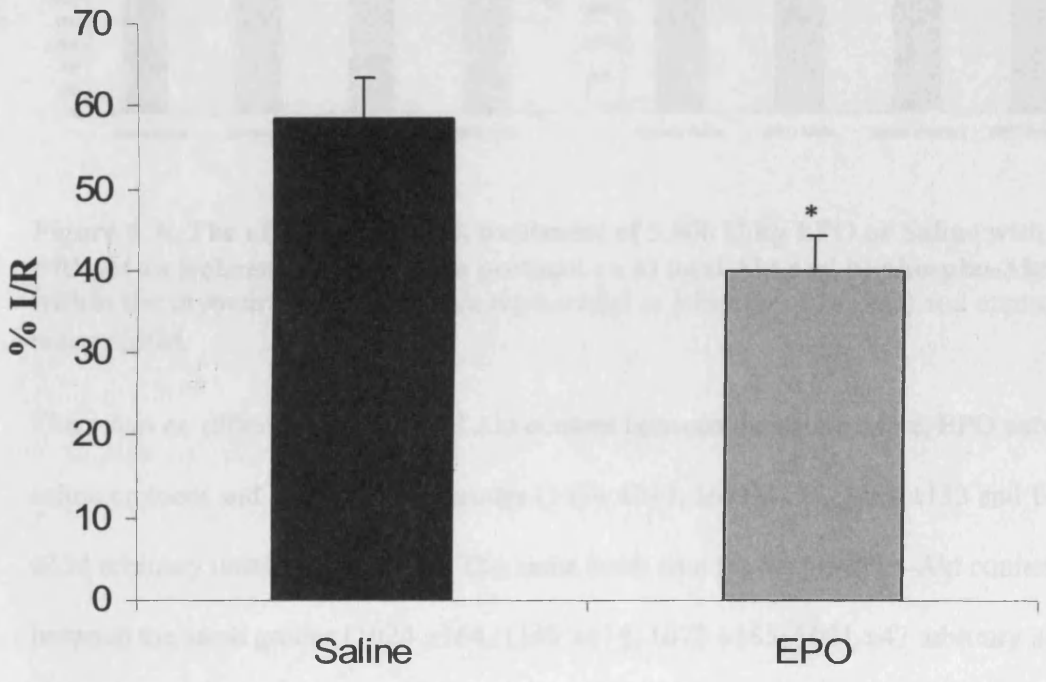


Figure 6. 5, The acute effect of EPO at a dose of 5000U/kg given 24 hours prior to index ischaemia. Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *p<0.05 vs control

An acute dose of EPO significantly reduced infarct size when compared to its control (IR% 39.9 ±4.4 v 58.4 ±4.9, p<0.05), see figure 6.5.

6.3.4 Western Blot Results

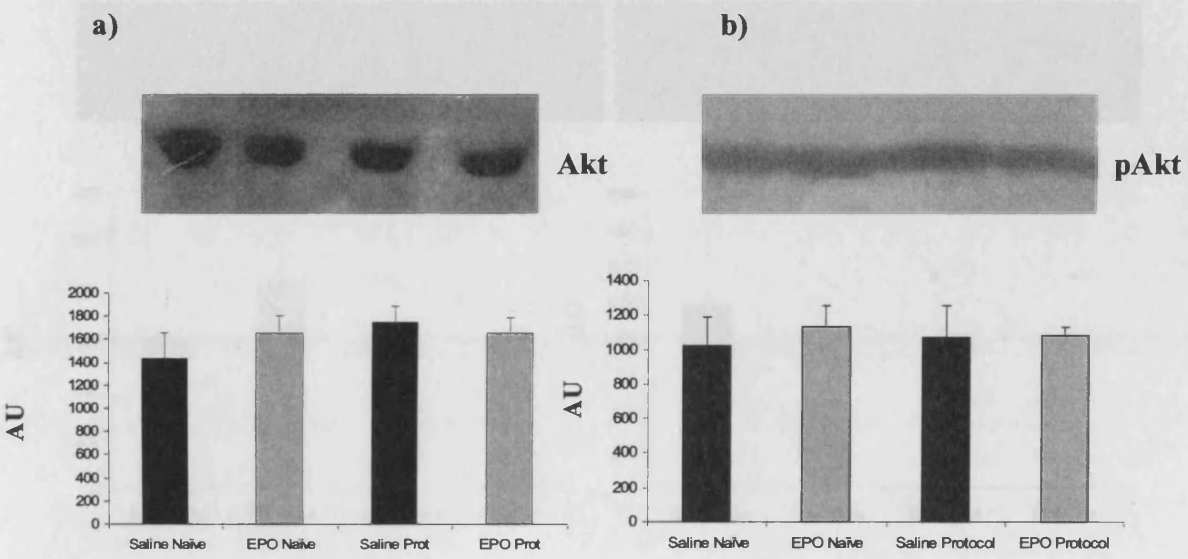


Figure 6. 6, The effect of a 3 week treatment of 5,000 U/kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total Akt and b) phospho-Akt levels within the myocardium. Values are represented as arbitrary units (AU) and expressed as mean ±SEM.

There was no difference in the total Akt content between the saline naïve, EPO naïve, saline protocol and EPO protocol groups (1434 ±214, 1651 ±154, 1750 ±133 and 1649 ±134 arbitrary units, respectively). The same holds true for the phospho-Akt content between the same groups (1024 ±164, 1139 ±114, 1072 ±185, 1081 ±47 arbitrary units, respectively).

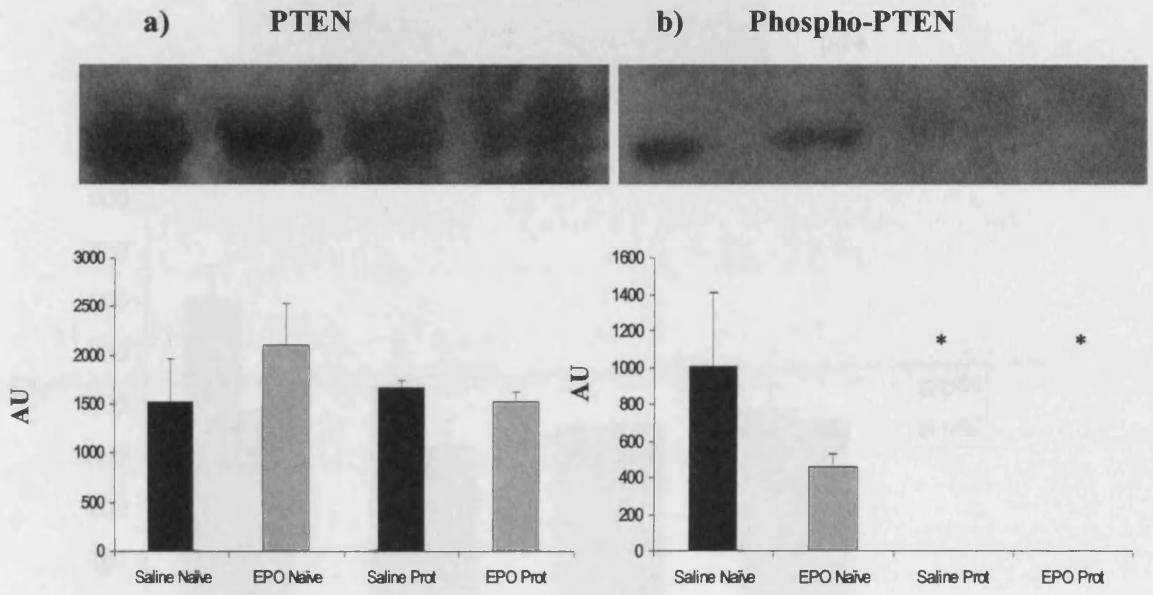


Figure 6. 7, The effect of a 3 week treatment of 5,000 U/Kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total PTEN levels and b) phospho-PTEN levels within the myocardium. Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ vs Saline Naive

In order to understand whether the PI3K pathway was being regulated by the enzyme PTEN (see discussion), we measured the total and phosphorylated levels of PTEN. As figure 6.7 shows, there was no difference in total PTEN content between the saline naïve, EPO naïve, saline protocol and EPO protocol groups (1534 ±429, 2111 ±416, 1676 ±61,

1531 ±96 arbitrary units, respectively). There was no significant difference in phospho-PTEN levels between the saline naïve and EPO naïve groups (1008 ±407 v 460 ±69, respectively). However both the saline protocol (0 ±0 v 1008 ±407 arbitrary units, $p<0.05$) and the EPO protocol (0 ±0 v 1008 ±407 arbitrary units, $p<0.05$) groups showed significant difference versus control.

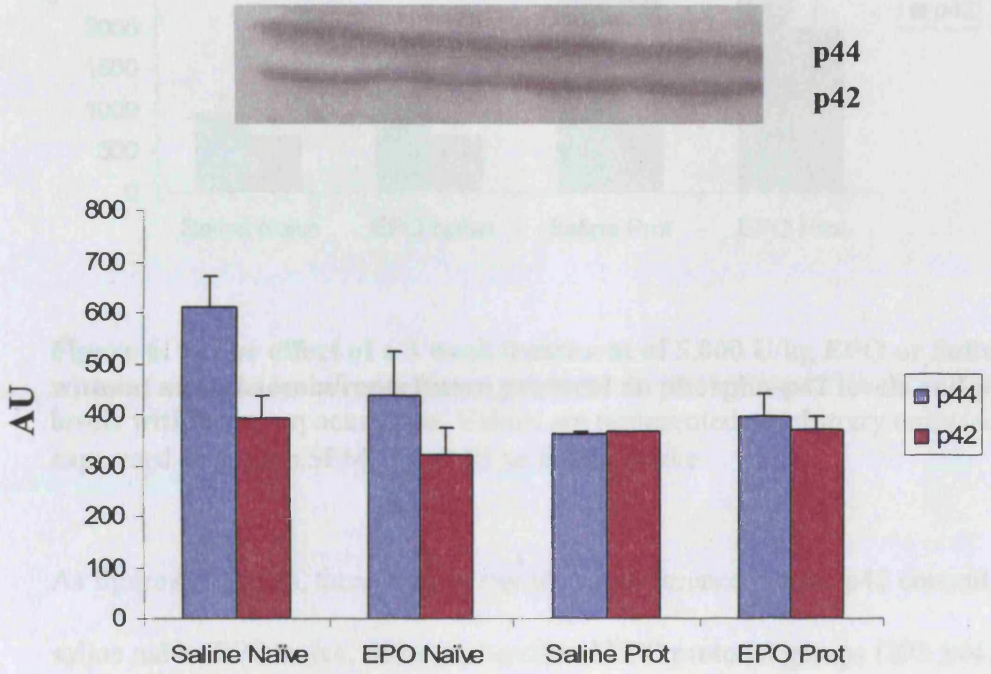


Figure 6. 8, The effect of a 3 week treatment of 5,000 U/kg EPO or Saline with or without an ischaemia/reperfusion protocol on total p42 levels and total p44 levels within the myocardium. Values are represented as arbitrary units (AU) and expressed as mean ±SEM.

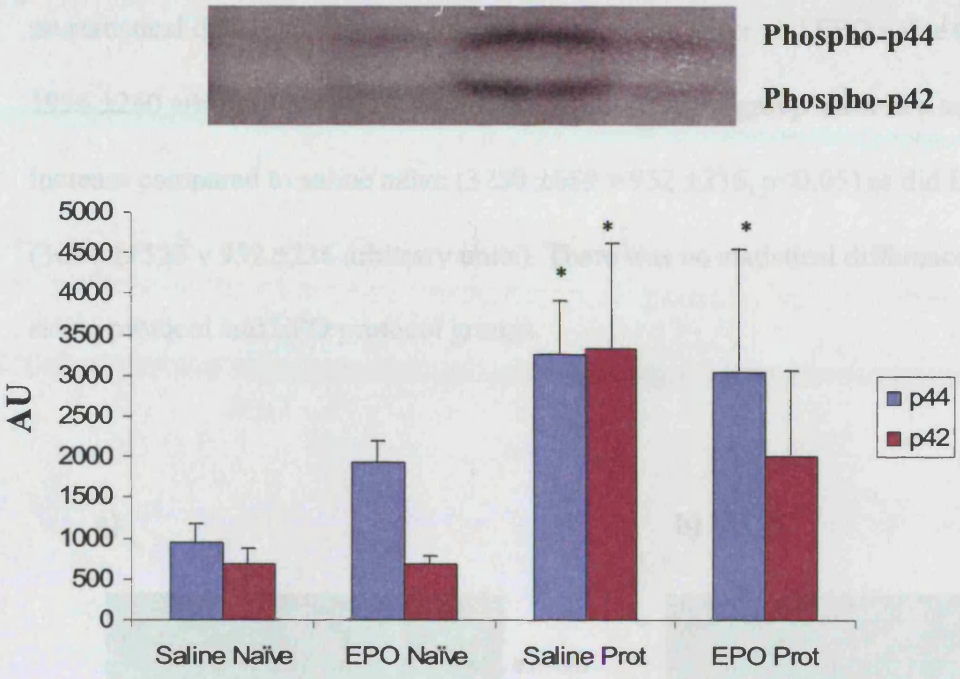


Figure 6. 9, The effect of a 3 week treatment of 5,000 U/kg EPO or Saline with or without an ischaemia/reperfusion protocol on phospho-p42 levels and phospho-p44 levels within the myocardium. Values are represented as arbitrary units (AU) and expressed as mean ±SEM. * $p<0.05$ vs Saline Naive

As figure 6.8 shows, there was no significant difference in total p42 content between the saline naïve, EPO naïve, saline protocol and EPO protocol groups (393 ±44, 320 ±54, 365 ±0.5, 371 ±25 arbitrary units, respectively). The same is true for total p42 content (610 ±61, 439, 439 ±84, 364 ±0.5, 396 ±46 arbitrary units, respectively). For phospho-p42 there was no significant difference between saline naïve, EPO naïve, and EPO protocol groups (692 ±178, 686 ±102, 1994 ±1075 arbitrary units, respectively). However, there was a significant difference between saline protocol versus saline naïve (3314 ±1309 v 692 ±178, arbitrary units $p<0.05$). There was no statistical difference between saline protocol and EPO protocol. With regard to phospho-p44 levels, there was

no statistical difference in these levels and the saline naïve and EPO naïve (952 ±236, 1936 ±260 arbitrary units). However, the saline protocol group showed a significant increase compared to saline naïve (3250 ±659 v 952 ±236, $p<0.05$) as did EPO protocol (3034 ±1526 v 952 ±236 arbitrary units). There was no statistical difference between the saline protocol and EPO protocol groups.

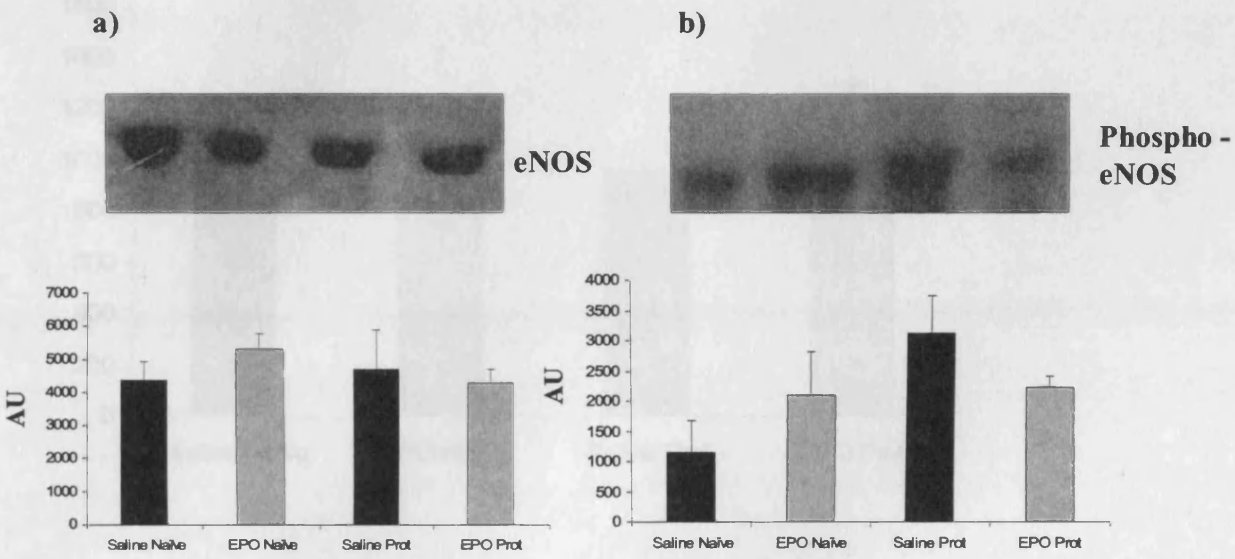


Figure 6. 10, The effect of a 3 week treatment of 5,000 U/kg EPO or Saline with or without an ischaemia/reperfusion protocol on a) total eNOS levels and b) phospho-eNOS levels within the myocardium. Values are represented as arbitrary units (AU) and expressed as mean ±SEM.

As figure 6.10 shows there was also no significant difference in total eNOS content between the saline naïve, EPO naïve, saline protocol and EPO protocol groups (4366 ±546, 5317 ±451, 4699 ±1170, 4299 ±379 arbitrary units, respectively). Likewise, there

was no significant difference between those same groups for phospho-eNOS (1143 ±531, 2085 ±730, 3113 ±618, 2225 ±172 arbitrary units, respectively).

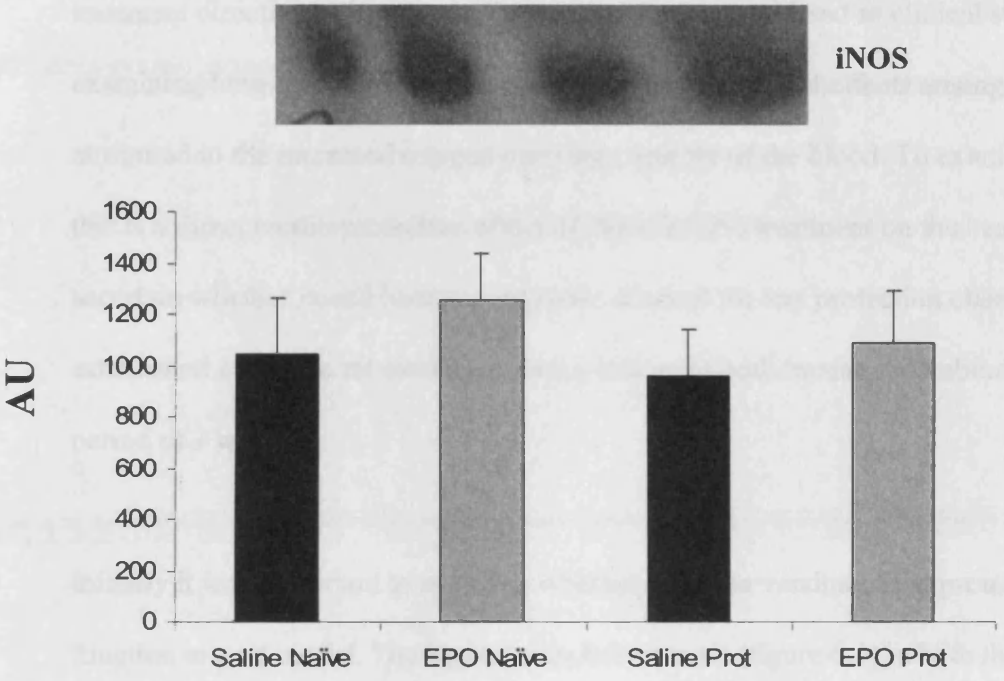


Figure 6. 11, The effect of a 3 week treatment of 5,000 U/kg EPO or Saline with or without an ischaemia/reperfusion protocol on total iNOS levels within the myocardium. Values are represented as arbitrary and expressed as mean ±SEM.

As figure 6.11 shows, there was no significant difference in total iNOS content between the saline naïve, EPO naïve, saline protocol and EPO protocol groups (1041 ±221, 1256 ±179, 957 ±186, 1087 ±205 arbitrary units, respectively)

6.4 Discussion

EPO is a drug currently in general clinical use to correct anaemia, with some patients on treatments lasting many years. Our study of EPO in a chronic setting, therefore, has potential clinical relevance. The potentially cardio-protective effect of chronic EPO treatment directly on the myocardium has not been considered as clinical studies examining long-term EPO treatment attribute any beneficial effects arising from EPO use attributed to the increased oxygen-carrying capacity of the blood. To examine whether this is a direct cardio-protective effect of chronic EPO treatment on the heart and ascertain whether raised haematocrit could account for any protection observed, we established a chronic rat model involving treatment with human recombinant EPO for a period of 3 weeks.

Initially it was important to establish whether a human version of the protein would function in a rat model. The increases in haematocrit (figure 6.1) in both the 'once-a-week' and '3 times-a-week' model clearly show that human EPO works in the rat. Thereafter, it was shown that a chronic treatment of EPO could limit myocardial injury induced by a standard Langendorff protocol of 35 minutes ischaemia and 2 hours reperfusion.

The ability of EPO to continue to protect the myocardium, shown in this chapter, after a period of chronic treatment is in some ways surprising. For example, atorvastatin, like EPO, is a drug that elicits protection via the PI3K/Akt pathway, but this protection is lost when atorvastatin is administered chronically and only restored with an additional acute

treatment[211]. In this way it is to be expected that, similarly, EPO's protective effects would be lost in a chronic-dosing regime. In support of our basic research data, there is clinical data suggesting that a chronic treatment with EPO, in order to correct anaemia, significantly improves the outcome of patients suffering chronic heart failure[133, 222, 223], even in patients where maximum tolerated therapy had previously failed to alleviate their chronic heart failure[222]. In addition, one study managed to reproduce this improved outcome in diabetic patients[224]. All these studies suggest that the effect we observe with EPO in the isolated perfused heart may be reproducible in the clinical setting in the patients at risk of myocardial infarction. It is worth noting that, in any clinical model, EPO would still have been present at the time of an ischaemic event, whereas in the animal model we use hearts that have been excised. Therefore, the EPO that was in the circulation is not present at the time that we make the heart ischaemic, but the protective effect remains in much the same way as EPO mimics preconditioning (see chapter 3).

6.4.1 The Role of PI3K/Akt in the Chronic Setting

As reported in chapter 4, EPO was able to limit infarct size in vivo and in vitro when administered at the point of reperfusion following a period of ischaemia in an Akt- and ERK-dependent manner. This involvement of PI3-kinase in mediating the protection of EPO in a cardiac setting has been reported by other groups[143, 144, 156] at a variety of time points and settings. It is thus interesting that wortmannin in our model should fail to abrogate the protection afforded by EPO in our chronic setting. One possibility to explain this finding is that the activation of PI3-kinase and Akt is a short-lived signal being regulated by phosphatases such as PP2A[225] and PHLPP [226]. The initial signal is sufficient

to provide acute protection, but is suppressed by phosphatases in the chronic setting. It has been shown in the past, within the myocardium, that eNOS is downstream of PI3K-Akt in an insulin-induced signalling cascade and confers a limitation in infarct size in an in vivo model of ischemia/reperfusion[227]. It is, therefore, possible that PI3K conferred activation of eNOS prior to its signal being suppressed, and that the eNOS activation was sufficient to provide long-term protection, also explaining the blocking effects of the NOS inhibitor L-NAME. An additional possibility, not examined in this study, is that the effect is mediated via PKC, which is known to be downstream of PI3K and to be activated by EPO[228]. In the previously mentioned study performed by Mensah et al[211], the authors found that atorvastatin could not protect in the chronic setting probably due to the elevation in levels of the phosphatase and tensin homolog deleted on chromosome ten (PTEN). The activation of PI3K can be beneficial in an acute setting by limiting cell death. However, prolonged activation of these protective pathways can have detrimental tumorigenic effects. In order to regulate the PI3K pathway, PTEN acts in equilibrium to the formation of the PI3K substrate PI-3,4,5-trisphosphate converting it back to PI-4,5ibiphosphate[229]. PTEN usually increases its activity by raising PTEN levels within the cell[230]. As can be seen from figure 6.7, there is no difference between groups in total PTEN levels. The role of phosphorylated PTEN is slightly ambiguous[231] but, again, as there is no difference between the groups, it could be argued that PTEN may be excluded from having any effect in this model.

6.4.2 The Role of Nitric Oxide in Chronic EPO Treatment

It has been shown that chronic treatment of endothelial cells with EPO can cause a marked rise in nitric oxide synthase (NOS) levels [232]. Furthermore, it has also been demonstrated, in rats, kept at high altitude, that they also had a raised haematocrit and increased iNOS levels[233]. As NOS has been implicated in protection from ischemia/reperfusion using other agents[234, 235], it is feasible to assume that NOS could be playing a mediatory role in this chronic setting. Interestingly, there has been one study examining the role of NOS that found EPO-mediated protection could not be blocked using L-NAME[153]. However, it should be noted that this study examined the role of NOS in an acute pre-treatment context. Therefore, it was important to determine the role of NOS in the chronic setting. The NOS-specific blocker L-NAME totally abrogated the reduction of infarction seen; this suggests that NOS does indeed play a key mediating role in protection as a result of chronic EPO treatment. In contrast, there were no significant observable increases in levels or activity of either eNOS or iNOS (see figures 6.10 and 6.10). There are a number of possibilities to account for this:

1. The Western blot is not sensitive enough to determine such subtle changes (as discussed in chapter 4)
2. The source of nitric oxide is nNOS (not measured in this study)
3. The difference between saline and EPO-treated groups is the location rather than level of NOS. For example, Gonzales et al managed to locate the increase in iNOS to the mitochondrial fraction[233]. Therefore, a localised increase in NOS within the mitochondria may not be detectable in the whole cell fraction.

It has been shown that nitric oxide inhibits mPTP opening in a number of studies[215, 235, 236]. There is some suggestion that a localised release of nitric oxide at the mitochondria[237, 238] may be protective against apoptosis by inhibiting mitochondrial respiration and free radical formation[239, 240], although it should be noted that Ghafourifar et al demonstrated that an increase in mitochondrial NOS (mtNOS) activity resulted in an increase in cytochrome c release[241]. Nitric oxide has been demonstrated to play an ambiguous role in apoptosis, capable of either preventing or inducing cell death[242]. We believe that in this setting NO plays an important anti-apoptotic role.

6.4.3 EPO-mediated Chronic Protection is Not Due to Raised Haematocrit

An 'acute' dose of EPO given 24 hours prior to index ischemia caused a limitation of infarct size compared to its saline control, despite the fact that there was no increase in haematocrit. Similarly, Xu et al demonstrated that administration of EPO 24 hours prior to index ischaemia elevated the heat shock protein HSP 70 and diminished the expression of NFkappaB, both phenomena have previously been associated with improved protection in ischaemia/reperfusion[243]. Additionally, in studies where EPO has been made non-erythropoietic by asialytion and carbamylation, protection was still observed [244, 245]. One explanation for this contradiction of absence of erythropoietic function, yet continuation of protection, is that the beneficial effect of EPO is mediated through its recently discovered effect on the common beta-receptor[124] rather than solely acting upon the EPO receptor. The common β receptor (βcR) subunit is known to increase affinity for such receptors as for granulocyte-macrophage colony-stimulating

factor (GM CSF) IL-3 and IL-5[246]. As βcR has been shown to functionally interact with the receptor[247, 248], it had been assumed that βcR would play a similar role for the EPO receptor. However, a knockout mouse model of βcR failed to eliminate the haemopoietic process suggesting that EPO was not functionally dependent upon βcR[249]. Brines et al subsequently established that the βcR heterodimerises with EPOR in cells that have been treated with EPO[124]. Finally, the authors demonstrated that carbamylated EPO (CEPO) failed to prevent staurosporine-induced apoptosis in βcR knockout mice, implying that the protective effects of EPO and CEPO are βcR-dependent and establishing a dual role for EPO.

The data presented in this chapter, in conjunction with the use of the blood free perfusion apparatus and the protective effects of non-erythropoietic EPO, suggests that the protection due to chronic administration of EPO is not due to any increase in oxygen supply to the myocardium from the blood.

In conclusion, our findings suggest that improved outcome of chronic heart failure patients undergoing chronic treatment with EPO may well be at least partially due to a direct effect on the myocardium via a nitric oxide-dependent pathway, rather than solely due to improved oxygen supply from the blood and that this direct effect on the myocardium could explain the beneficial effects seen by EPO in clinical studies.

Chapter 7 - The Effect of EPO when Administered After Reperfusion

7.1 Introduction and Aims

Reperfusion is necessary to salvage the myocardium subsequent to a period of ischaemia but, paradoxically, can itself cause additional injury, as discussed in detail in chapter 3. However, in unstable angina the thrombus may have partially disintegrated, allowing reperfusion before medical attention can be received by the patient. It has been shown that, if putative protective agents such as insulin have their administration delayed by 15 minutes, then it is not possible to protect the myocardium[69]. Thus, in a clinical scenario whereby reperfusion has already commenced, the protective adjunct is ineffective. EPO has been shown to be protective when administered by intra-peritoneal injection 5 minutes into reperfusion in an in vivo rat model which included a 24 hour period of reperfusion[183]. Intra-peritoneal injections are considered to take approximately 10 minutes to enter the bloodstream, so it could be argued that the time of EPO administration was 15 minutes after reperfusion had commenced. Based upon this study we hypothesised that we could protect the myocardium in an in vitro model of ischaemia/reperfusion by administering EPO subsequent to the commencement of reperfusion.

7.2 Experimental Protocol

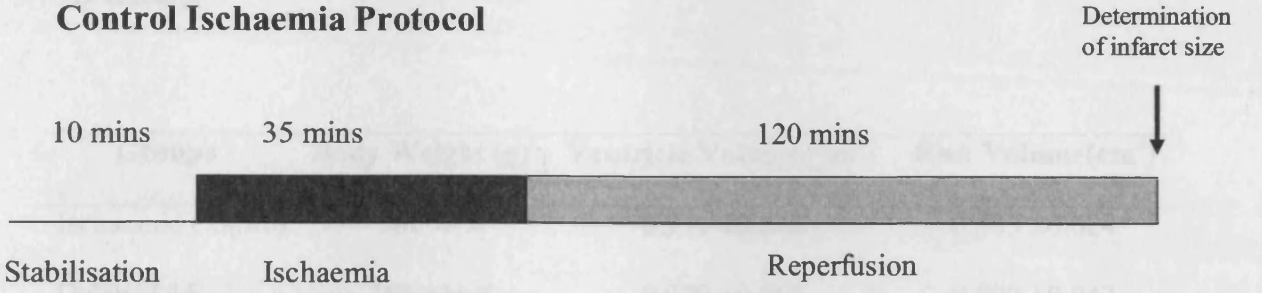
Hearts from rats weighing 300-400g were excised and mounted on a Langendorff perfusion apparatus as described in chapter 2. The following protocols were used:

1. Ischaemic control – standard 35 minutes ischaemia and 2 hours reperfusion with no treatment (n=7)

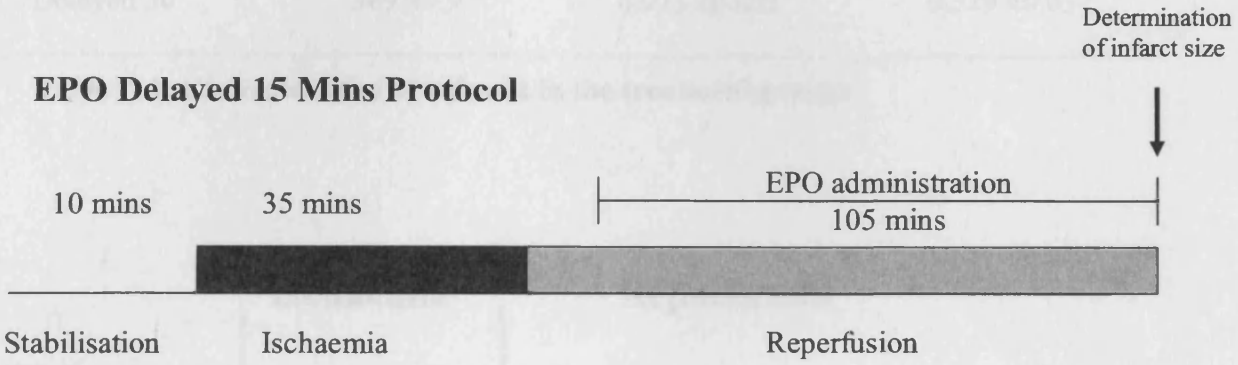
2. EPO 15 – EPO administered 15 minutes after commencement of reperfusion and continued throughout the reperfusion period (n=8)

3. EPO 30 - EPO administered 30 minutes after commencement of reperfusion and continued throughout the reperfusion period (n=8)

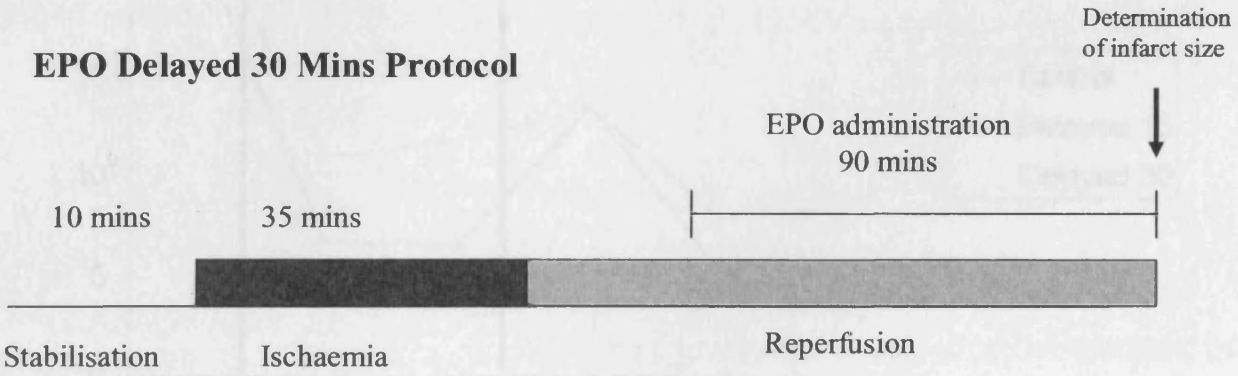
Control Ischaemia Protocol



EPO Delayed 15 Mins Protocol



EPO Delayed 30 Mins Protocol



7.3 Results

Groups	Body Weight (g)	Ventricle Volume (cm^3)	Risk Volume(cm^3)
Ischaemic Control	361 ±6.6	0.979 ±0.075	0.443 ±0.024
Delayed 15	358 ±15.5	0.979 ±0.051	0.500 ±0.047
Delayed 30	369 ±7.9	0.973 ±0.032	0.529 ±0.037

Table 7. 1, Characteristics of animals in the treatment groups

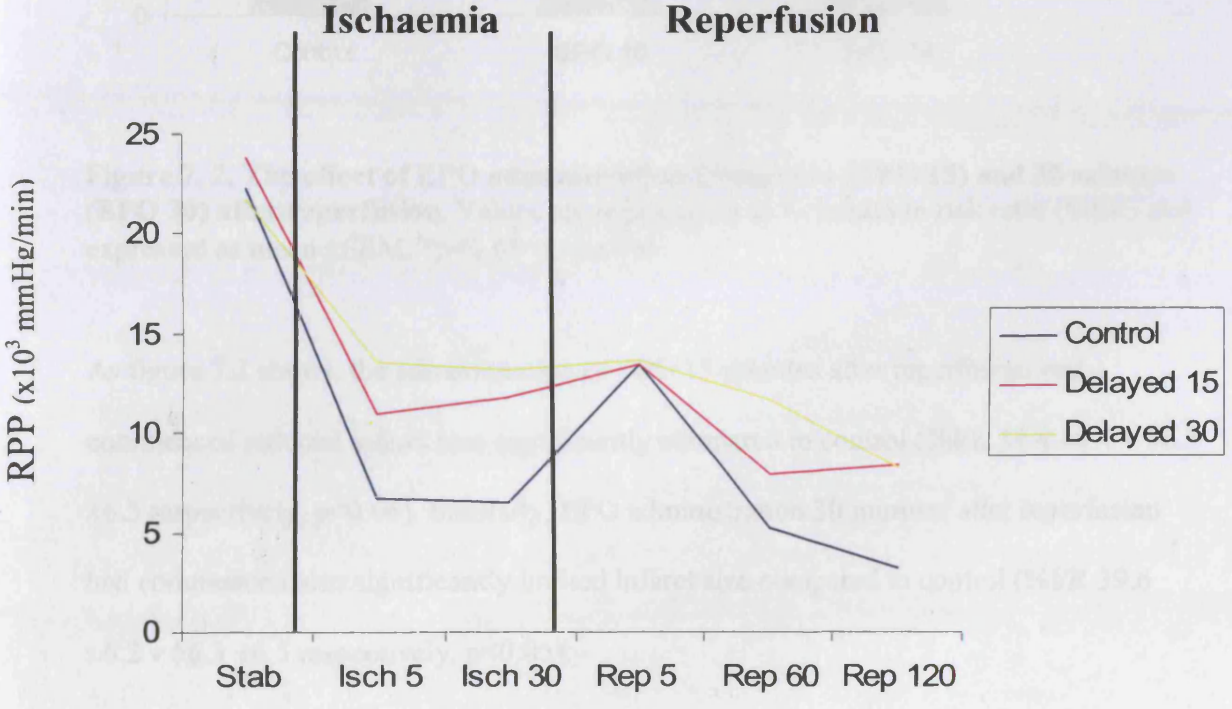


Figure 7. 1, Representation of the change in Rate_Pressure Product (RPP) throughout the experimental protocol from stabilisation (Stab) through ischaemia (Isch) and into reperfusion (Rep)

Exclusions

There was one exclusion from the EPO 15 group due to too large a risk zone.

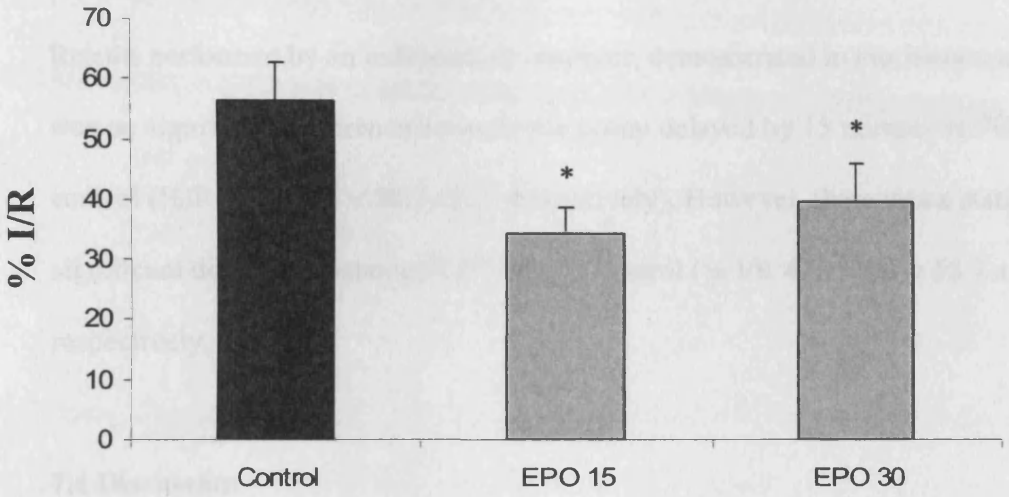


Figure 7. 2, The effect of EPO administration 15 minutes (EPO 15) and 30 minutes (EPO 30) after reperfusion. Values are represented as % infarct to risk ratio (%I/R) and expressed as mean ±SEM. *$p<0.05$ vs control

As figure 7.2 shows, the administration of EPO 15 minutes after reperfusion had commenced reduced infarct size significantly compared to control (%I/R 34.4 ±4.4 v 56.3 ±6.5 respectively, $p<0.05$). Similarly, EPO administration 30 minutes after reperfusion had commenced also significantly limited infarct size compared to control (%I/R 39.6 ±6.2 v 56.3 ±6.5 respectively, $p<0.05$).

Results performed by an independent observer, demonstrated in our laboratory that there was no significant difference between the group delayed by 15 minutes (EPO 15) and control (%IR 49.2 ±5.4 v 58.7 ±5.7, respectively). However, there was a statistically significant difference between EPO 30 and control (% I/R 42.1 ±4.1 v 58.7 ±5.7, respectively, $p<0.05$)

7.4 Discussion

Lipsic et al found in an in vivo rat model of ischaemia/reperfusion that administering EPO at different time points from pre-ischaemia to 5 minutes into reperfusion provided very similar reductions in infarct size compared to control (from a 19 to 23% reduction)[183]. As the injection given at reperfusion was intra-peritoneal, it can be assumed that the time to absorption into the bloodstream was approximately 10 minutes, making the actual timing for administration 15 minutes after reperfusion has commenced. The data presented in figure 7.2 indicates that EPO does indeed reduce injury when perfused 15 minutes into reperfusion, although not by as much as when administered prior to ischaemia or at the point of reperfusion, see chapters 3 and 4. Indeed even delaying administration until 30 minutes into reperfusion still provides a significant level of protection. The alternative observer noted a significant reduction in infarct size when

EPO was given 30 but not 15 minutes into reperfusion. This may seem counter-intuitive but may just be a function of the comparatively low levels of protection presented in this setting and thus requires additional numbers to clarify whether protection is present in that model.

As a general rule it is considered necessary to administer any protective agent within 15 minutes of reperfusion if any limitation of injury is to be observed[69]. The data presented within this chapter seems to be at odds with this paradigm. However, there is evidence within the literature that supports the theory that treatment later into reperfusion can still be protective. The Lipsic data[183] and that provided by the alternative observer suggest that the original results obtained may represent a real occurrence. A greater level of protection may have been seen by Lipsic et al as a result of their extended, 24 hour period of reperfusion indicating that not only is cell death on-going, but that injury occurring some time after ischaemia/reperfusion is salvageable. In addition, further supporting data is provided by Paul Govewalla from our laboratory. He observed in an in vivo recovery model of rat myocardial ischaemia reperfusion [191] that, when he co-administered the PI3K blocker, LY294002, with EPO, there was only a partial reduction in EPO-mediated protection. When an alternative PI3K blocker, wortmannin, was co-administered with EPO, the protection was completely abrogated. The explanation for this difference in results could lie in the reversible nature of LY294002[250]. It may be assumed that, as soon as the reversible inhibitor was washed out from the system, EPO, which was still present in the circulation, could activate protective mechanisms inducing the partial results observed. In other words, the irreversible wortmannin does not allow any later activation of PI3K and thus protection was completely abrogated. It is possible that the later

activation of PI3K, in animals treated with LY294002, would explain the partial protection induced by EPO in this group.

Solenkova et al conducted a series of experiments where they used wortmannin at differing points in reperfusion in preconditioned hearts. They found that they could block the effects of preconditioning by administering wortmannin as late as 30 minutes into reperfusion[251]. The fact that PI3K is still having an active protective effect at 30 minutes after reperfusion lends support to the data presented in this chapter. The important question, however, is how the protection is manifest.

7.4.1 A New Paradigm for Myocardial Salvage?

The ability for EPO to reduce infarct size at a much later stage in the apoptotic/necrotic process than any other agent thus far studied asks the question: at what point in the apoptotic/necrotic process does the cell pass the point of salvage? Previously, it had been considered to be the point at which the mPTP opens, mitochondrial membrane potential collapses and cytochrome c is released. This pore opening occurs within a few minutes of the commencement of reperfusion[252, 253]. Therefore, an ability to protect when given at a much more delayed time-point suggests that pore opening by itself cannot irreversibly lead to cell death. It is known that EPO limits apoptosis within erythroid precursor cells in the haemopoietic process by the inhibition of caspases, see chapter 1 [254]. This inhibitory effect on caspases is not limited solely to erythroid precursor cells as EPO has also been shown to inhibit caspases 1, 3, 8 and 9 within neuronal cells, preventing injury in the process[255, 256]. Inhibition of caspases has also been shown previously to have a protective effect upon infarction in an ischaemic/reperfused isolated rat heart[14].

Therefore, at this stage we cannot be confident that this delayed protection is due to the effect of EPO upon the inhibition of caspases or upon the activation of the pro-survival kinases PI3K/Akt, or both.

7.4.2 Clinical Implications of EPO-mediated Protection after Reperfusion

EPO could be given in conjunction with a thrombolytic agent in instances where the patient undergoing an acute myocardial infarction (AMI) is also suffering from anaemia. By comparison, insulin is also routinely administered in AMI patients that have high glucose levels. In a number of studies examining the effect of insulin given in combination with glucose and potassium (GIK) at reperfusion, it has been found that GIK infusion has a positive effect in improving outcome and prognosis in patients[257-260]. However, in the more recent CREATE-ECLA trial conducted in South America and India, GIK was found to have no positive effect compared to control. The reasoning behind this failure was due to the possible delay in administering GIK[261], thus indicating the narrowness of the time-window available after reperfusion in order to observe any protection. In basic research studies, the narrow time-window is also seen in the study where insulin administration was given within 15 minutes after reperfusion had started with no protection observed[262]. However, the data presented in this chapter suggests that even a later treatment of EPO, *after* the commencement of the reperfusion, might still provide a significantly better outcome for the patient.

The ability to prevent cell death at a much later point in the cell death process than other beneficial drugs could have the effect of being able to improve the outcome and

prognosis of patients that have had a delay in receiving medical attention. This added ability of delayed protection from infarction gives EPO a potential advantage in the clinical setting compared to other putative protective agents.

Chapter 8 - Summary, Conclusions and Future Directions

The primary aim of this thesis was to examine the potential protective effects of EPO in a variety of myocardial ischaemia/reperfusion settings and to examine the mechanisms by which such protection could occur. The central focus was related to the mechanism and involved an investigation of the PI3K/Akt, ERK pathway and mPTP as well as the isoforms of NOS, which have been demonstrated to induce protection against the ischaemia reperfusion injury and also to be cellular targets for EPO in many cell systems.

8.1 Summary of Findings

8.2.1 EPO as a Preconditioning Mimetic

Previous studies involving EPO-mediated protection within the myocardium have used models in which EPO was still present at the commencement of index ischaemia. The data presented within this thesis demonstrates for the first time that EPO is able to exert a protective "memory" effect within the heart and appears to mimic the phenomenon of ischaemic preconditioning. In a manner similar to ischaemic preconditioning (IPC) our data also demonstrates that EPO appears to use the PI3K signalling pathway and not ERK 1/2 to achieve this protection. This initial study confirms the protective effects of EPO when administered pre-ischaemicly to the heart, in which a significant reduction of infarct size was observed. Interestingly, previous studies from other laboratories have also demonstrated an ability for EPO to mimic preconditioning in a neuronal model of ischaemia/reperfusion[129].

8.2.2 EPO at Reperfusion

Recent studies have shown that administration of EPO when given at reperfusion limits injury within the myocardium. We have reproduced the data within a Langendorff perfusion model demonstrating that a 50ng/ml, or 10U/ml, is the most effective dose. This protection was seen to be mediated by PI3K, ERK 1/2 and eNOS, as demonstrated by the ability of PI3K inhibitors wortmannin and LY294002, the ERK inhibitor and the NOS inhibitor to abolish EPO-mediated protection. However, Western blot analysis of protein kinase activation proved problematic in that it proved difficult with this technique to demonstrate activation of protein kinase members of the putative protective pathways. However, eNOS was shown to be activated subsequent to EPO perfusion within the heart and may account for some of the protection observed.

8.2.3 EPO within a Cellular Model

EPO-mediated protection in the heart has been associated with a delay in mPTP opening. These findings were confirmed in this thesis using a myocyte model of pore opening that utilised oxidative stress produced from laser excitation of TMRM pre-loaded into the cell. This protective effect on the mPTP was found to be dependent on PI3K and NOS as wortmannin and L-NAME abolished the beneficial effect observed with EPO. The ERK 1/2 inhibitor U0126 had the unexpected effect of strongly delaying pore opening in its own right. It seems likely that this outcome of U0126 may be artefactual and specific to this cellular model as U0126 has not been seen to be protective in any other myocardial model of ischaemia/reperfusion.

8.2.4 A Model of Chronic EPO Treatment

Sprague Dawley rats were treated for 3 weeks with EPO in order to try and produce a more clinical scenario similar to that observed when patients receive a chronic treatment with this drug. A once-a-week treatment of 5,000 U/Kg of EPO failed to reduce infarct size in hearts that had been excised and underwent a standard protocol of ischaemia and reperfusion in a Langendorff model of heart perfusion. A three times-a-week treatment did, however, significantly limit injury of hearts in the same model. This protective effect was abolished by the NOS inhibitor L-NAME but not the PI3K inhibitor wortmannin. Western blot studies highlighted the absence of a raised level of PTEN, which is normally associated with longer-term activation of PI3K in order to repress the PI3K signal which chronically could be detrimental[263]. No elevation in levels or activation of NOS, Akt or ERK was observed. In order to eliminate the possibility of raised haematocrit as a possible mediator of protection, EPO was administered 24 hours prior to index ischaemia and was also shown to protect in the same manner without any raised haematocrit.

8.2.5 EPO Protects After Delayed Administration in Reperfusion

It has been shown that it may be possible to limit infarct size when agents are given up to 15 minutes of reperfusion[69]. However, a study by Lipsic et al also demonstrated that it was possible to protect the myocardium when EPO was given after 15 minutes of reperfusion[183]. The data in this thesis also showed that EPO could limit infarct size even when given up to 30 minutes into reperfusion and may represent an important aspect of the drug's pharmacology. However, an in-depth study of the potential mechanism associated with this delayed effect needs to be undertaken.

8.3 Conclusions

In the last 3 years EPO has become well established as a potential cardio-protective agent both in basic research and clinical studies. Within this thesis we have shown that EPO is beneficial in a variety of settings, time-points and models. The method by which EPO protects seems to be multi-faceted, i.e. using multiple pathways, and depends upon the time of administration. PI3K is an important mediator of all acute methods of administration of EPO but appears to become more tightly regulated in the chronic setting. NOS is the one ubiquitous factor seemingly involved at every stage of EPO treatment from acute to chronic administration, although different NOS isoforms may be responsible for eliciting protection in these settings. ERK 1/2 seems to be only involved in EPO-mediated protection when administration occurs at the time of reperfusion.

As observed in the literature, EPO seems to activate a series of beneficial signalling pathways, from PKC to caspase inhibition, all of which may explain the potential beneficial effects of this agent in such a wide range of settings, not just within models of ischaemic heart disease but also within a whole host of differing pathologies.

8.4 Potential Clinical Implications

8.4.1 Elective Surgery

In circumstances where elective surgery involves periods of myocardial ischaemia, pre-treatment of EPO could limit any injury caused by the ischaemic period and the subsequent reperfusion of the tissue. There are reports that EPO also has the effect of

damping down inflammation[264, 265], which would have an additive beneficial effect to that directly on the cell within this clinical setting.

8.4.2 Emergency Intervention

With the strong protective effect of EPO given at reperfusion and the ability to protect even when administration is delayed, EPO could be singularly effective as an agent to be used in emergency acute myocardial infarctions in conjunction with a thrombolytic, particularly in those patients who have not received prompt medical intervention.

8.4.3 General Treatment of Heart Disease

A series of clinical studies have shown that treatment of patients with chronic heart disease suffered reduced hospitalisations, ischaemic events and a generally improved quality of life when treated with EPO. EPO intervention was even effective within aged patients and those with diabetes. With the advent of non-erythropoietic versions of EPO, the problematic side-effects of EPO, namely thrombus formation due to blood thickening, should be circumvented making the compound safe to use with non-anaemic patients.

8.5 Limitations and Further Investigations

The thesis did not and could not cover all aspects related to the involvement of EPO in preventing ischaemia/reperfusion injury. However, the data presented opens up other avenues of interest for further research:

8.5.1 Preconditioning Studies

Activation of NOS has been shown to limit injury in other settings of ischaemia/reperfusion. Could it also mediate protection when EPO is administered prior to index ischaemia?

IPC was shown to be abolished when various kinase inhibitors were administered at reperfusion. Can this effect be repeated in EPO-mediated mimicry of preconditioning?

8.5.2 EPO at Reperfusion

The Western blot experiments within this study did not show any increase in activation of target kinases. The blots should be repeated for different timepoints in reperfusion in order to establish whether there is a window of activation.

In addition, it would be important to establish the source of the NOS involved in EPO-mediated protection, i.e. is the NOS responsible located at the mitochondria?

8.5.3 EPO Within a Cellular Model

The ability of U0126 to strongly delay mPTP opening in myocytes needs to be examined. Will a different ERK inhibitor have the same effect suggesting that ERK inhibition does indeed delay pore opening? Or will U0124, which is structurally similar but inactive, also delay pore opening, suggesting that the phenomenon is artefactual?

It was not established in this study which isoform of NOS was mediating the delay in pore opening provided by EPO. The location of the enzyme, cytosol or mitochondria, also needs to be determined.

There has been some suggestion that the connecting point between protein kinases and prevention of mPTP opening could be mediated by GSK 3β. The involvement of GSK 3β in the EPO-induced protective pathways in this model needs to be established.

8.5.4 Chronic EPO Treatment

It has been suggested that rats maintained at high altitude show increased levels of mitochondrial NOS. Is there a similar increase in mtNOS levels in rats chronically treated with EPO? Is mtNOS responsible for mediating protection in a chronic EPO model?

Also, EPO still managed to afford protection after chronic treatment. Have the phosphatases that regulate such key kinases as ERK and PI3K/Akt been inactivated?

8.5.5 EPO at Delayed Reperfusion

This thesis demonstrated that EPO could limit protection even when administered 30 minutes after the commencement of reperfusion. The maximum length of possible delay prior to EPO administration needs to be established.

The experiments in this study need to be repeated in an in vivo model with an extended reperfusion protocol to ensure that this ability to protect after a delay in administration occurs within a whole animal. The extended reperfusion period will establish whether

any protection seen is merely a temporary delay in cell death or permanent salvage of tissue.

Furthermore, the protective pathways that mediate protection in this model need to be established by using pharmacological inhibitors in conjunction with EPO.

Using a pan-caspase inhibitors would establish whether inhibition of caspases is sufficient by itself to limit injury at this delayed.

If EPO is able to prevent cell death in cells where the mPTP has already been opened prior to administration, is it possible to prevent cell death in myocardium where the mPTP has been forcibly opened pharmacologically, e.g. by atractyloside?

Publications and Communications

Bullard, AJ, Yellon, DM (2003) *Erythropoietin protects the myocardium from ischaemia/reperfusion injury*. University College London Cardiovascular Science and Medicine Meeting, Institute of Child Health, London.

Bullard, AJ, Govewalla, P, Yellon, DM, (2004), *Erythropoietin protects the myocardium from ischaemia/reperfusion injury*. November 2004, New Orleans meeting of the American Heart Association.

Bullard, AJ, Govewalla, P, Yellon, DM, Erythropoietin protects the myocardium against reperfusion injury in vitro and in vivo, *Basic Res Cardiol.* 2005 Sep;100(5):397-403.

Bullard AJ, Yellon, DM, Chronic Erythropoietin Treatment Limits Infarct-size in the Myocardium in Vitro, *Cardiovasc Drugs Ther.* In press

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